# BIOFUNCTIONAL MICROSTRUCTURED SILICA COATING ON DENTAL MATERIALS

Alejandro Peláez Vargas. DDS. BMEs. M.Sc.

*Dissertation compiled to fulfill partial requirement for the degree of "Doctor of Philosophy" of the Doctoral Program in Biomedical Engineering*

Supervised by
Fernando Jorge Mendes Monteiro. Eng. M.Sc. Ph.D



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# DEDICATION

JuanDa y Abigaíl su amor, energía positiva y apoyo
son el sustento en todos los instantes de mí vida.
Infinitas gracias por celebrar conmigo cada
pequeño logro y por ayudarme a encontrar
puertas abiertas en los momentos
más difíciles.

Analieze mil gracias por ayudarme
a luchar contra mí
minimalismo.

A todos los amigos y sus familias que me convirtieron en padrino, tío putativo, hijo mayor, hijo menor o huésped especial Duránte estos seis años. Espero que sepan de mí eterno agradecimiento.

Alejandro Peláez Vargas

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# THE PAST, PRESENT AND FUTURE OF AN IDEA FOR MY PhD RESEARCH

The main reason to include this section in this work, is to give to my thesis a personal touch. In the scientific activity, like in other human tasks, always we would find some cultural aspects, skills, weaknesses, and people that help or hinder us in the development of our projects. Day by day we face these factors and we need to make options to prevent them to turn into obstacles. However, these experiences are generally lost and forgotten in the formal and rigid scientific language in a Ph.D. dissertation. This section may be considered a tribute to many hours of discussions with friends, colleagues and relatives, who contributed in one way or another, for reaching a goal at the end.

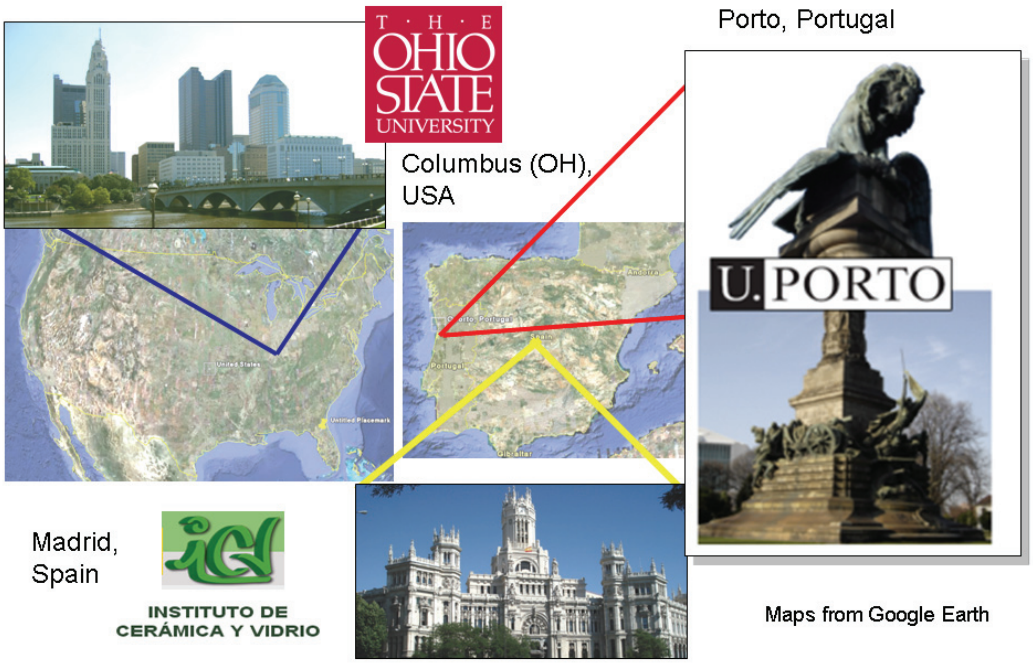



Figure 1. Where this work was conducted?

Representative images from main places where the present work was made. Image from Boavista Square at Porto (Portugal), Building of Spanish Bank at Madrid (Spain) and the down town at Columbus (Ohio, USA).

*Context*

The genesis of the present work was originated in friendship between many people, some of these collaborators might be described in continuation: As historial background, in 1984, nineteen countries (Argentina, Bolivia, Brasil, Chile, Colombia, Costa Rica, Cuba, Ecuador, El Salvador, España, Guatemala, Honduras, México, Nicaragua, Panamá, Paraguay, Perú, Portugal, Republica Dominicana, Uruguay and Venezuela), created an iniciative for multilateral cooperation called: "Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo - CYTED". Several networks have been created based in this program, but three networks were essentials in the concretization of the present work: The network number VIII.J "Red Iberoamericana de Biomateriales para la Salud" (Started: 2001 Finished: 2004), leader by Professor Roberto Sastre (deceased), network number VIII.E "Red Iberoamericana sobre Ciencia y Tecnología de Materiales Vítreos" (Started: 1997 and finished: 2001) leader by Professor Alicia Durán and network VIII.9 "Desarrollo de revestimientos vítreos funcionales" (started:1999, finished: 2002) leader by Professor Pablo Galliano.

These networks left an harmonic friendship between Prof. Pablo Abad (Universidad Nacional de Colombia, Medellín, Colombia), and Professor Alicia Durán (Instituto de Cerámica y Vidrio, CSIC, Madrid, Spain) and Professor Fernando Jorge Monteiro (Universidade do Porto, Porto, Portugal).

The word "Sol-gel" to protection of biomedical materials from degradation in the scientific program of these networks based in the work of Professor Durán's Group. Initially, Professor Claudia García, a researcher from the Professor Abad's Group and former PhD student from Durán's Group, published some works, where the bio-activation of metallic substrates (AISI 316L stainless steel, Ti6Al4V or cobalt-chromium alloy) was described using glass thin films and bioactive particles of glass, glass ceramic and

hydroxyapatite. In parallel, I was working in my master dissertation at Universidad Nacional de Colombia (supervised by Professor Pablo Abad and Professor Maria Elena Márquez) on the genotoxicity approach of these bioactive thin films applied on AISI 316L stainless steel. In 2003, we obtained the financial support of Colciencias for the project entitled "Análisis semiautomático de la citotoxicidad y genotoxicidad *in vitro* de recubrimientos bioactivos", an important experience for the follow years.

In 2004, supported by the previously introduced project, I was training for short time in Professor Monteiro's Group. This training served to define a new project to participate in the call for proposals for PhD scholarships from Alban Program in the same year. In the formulation of a new project Professor Monteiro made some conditions such as 1) to work in a field near of his Group, 2) to work on cell-biomaterial interface and 3) to work on a clinical dentistry problem. Initially, we decided to use the combined approached including glass thin films and bioactive particles to improve the ceramic surface of all-ceramic dental abutments.

In 2005, after going back home and considering the possibility that our project could be refused by the Alban Program. Professor Luis F. Restrepo, Professor Gabriel J. Gallego and I had been working to start the new area of work on dental ceramics in the CES-LPH research Group in the School of Dentistry at Universidad CES, where I was working as an Assistant Professor from 1997. After, this area was successfully started, we tried to extend this idea to an inter-institutional cooperation. Based in my privileged position in the context of other regional Universities such as Universidad Pontificia Bolivariana (Coordinator of Master Program in Biomedical Engineering), Universidad de Antioquia (Invited Professor in the Postgraduate Program in Dentistry) and Universidad Nacional de Colombia (Researcher in Professor Abad's Group).

*Context*

The formation of an inter-institutional group was a very interesting but unsuccessful initiative. However, it was a good attempt to believe in a commercial slogan "Nothing is impossible". Some bad experiences might be explained here but I prefer to say that in this experience it was possible to define the topic of the work with many specialists and graduated students, some of them are co-authors of papers based on different sections of the present dissertation. (i.e. Dr. Jaime Dussán, Dr. Santiago Arango, Dr. Juan Carlos Cuartas and Dr. Laura Osorio).

In 2006, after several complicated process to obtain the Portuguese visa, I was finally in Porto (Figure 1). The first year was invested to complete the physico-chemical characterization of different products related with dental ceramics obtained in the 2005, under supervision of Prof. Monteiro.

In 2007, I started the training on primary cultures in the Professor Maria Helena Fernandes' Group at the Faculty of Dental Medicine at University of Porto (She never will understand her true contribution in my formation. Several times we did not share the same point at view). In the middle of 2007, some friends changed the direction of my dissertation, when they introduced me for two techniques: 1) Statistical Experimental Design and 2) Micro- nanofabrication. These techniques had been used by Herney Ramírez (former PhD Student at University of Porto) and Daniel Gallego (former PhD Student at The Ohio State University), in their respective research groups.

These techniques changed the direction of my dissertation because the Statistical Experimental design helps to optimize multivariable processes using a reduced amount of experiments and the microfabrication based on soft lithography helps us to produce engineered surfaces that promote tissue

guided regeneration. Both techniques helped us to improve the initial project presented to Alban Program scholarship program. The up-graded project was presented for a scholarship call of the Portuguese Science Foundation in the same year.

This project obtained a favorable evaluation and two important situations then appeared: 1) To start the collaboration between Prof. Monteiro's Group and Prof. Hansford's Group and 2) To extend my PhD training for four years more, including a long term stay in Dr. Hansford's group. In October of the same year, Prof. Hansford and his PhD Student Nicholas Ferrell came to Porto to establish a more realistic collaboration with Prof. Monteiro. Subsequently, Nicholas Ferrell made a short visit at INEB and I did the first training on microfabrication and we made the first prototypes of micro-fabricated silica coating modified with bioactive particles.

2008 was a year where three important things happened: 1) I was trained in processing yttria stabilized zirconia in the Prof. Aza's Group at Instituto de Cerámica y Vidrio de Madrid (Figure 1). 2) I completed the transition from Alban Program to the FCT scholarship and 3) Professor Monteiro and his partners, had the "Bonamidi"project approved by FCT, in which several new approaches were included as animal characterization and *in vitro-in vivo* noninvasive human evaluation. The most important achievement obtained with this project was the consolidation of an international network with different possibilities for all the participants from Portugal, Spain, USA and Colombia.

In 2009, I went to work in the Professor Hansford Group at The Ohio State University (Figure 1) to obtain training in microfabrication. It was a very important experience because during this period I found for first time a group of Ph.D students (Eng. Daniel Gallego and Eng. Natalia Higuita) dedicated to

work in the same area as myself. It was the more successful period in the doctoral training in terms of scientific production, creativity and positivism. Finally, the participation of the environment of Nanotech West Laboratory was fundamental for this success.

Since 2010 until now, after caming back to Porto, the Bonamidi project started and at the same time, I was trying to complete the minimal publication requisites before to deliver the present work. This caused to me a lot of uncertainty because the minimal publication requirements are not clearly defined. The application of this criterion is very heterogeneous. After 18 months of hard work and hopes, this work has come to an end. I must include that this period helped me to obtain introductory information to add two new techniques to my background: microfluidics and time-lapse Microscopy, thanks to Prof. Hansford (Research visiting Prof. Monteiro's Group) and Dr. Sampaio respectively.

In the last 18 months, the permanent scientific discussion with friend/colleagues as Eng. Angela Carvalho and Microbiologist Liliana Grenho (members of Bonamidi) was wonderful. They helped me to think about new ideas in microfabrication applied in Dentistry.

What next? … The future is still open. I hope to maintain the contact with all people in the scientific network described previously and to develop some ideas for new guided regeneration, in my country, close of my family.

# HUMAN RESOURCE IN THE PRESENT WORK

All people involved in some phase of the present work is described in the next section:

| Name | Function/Activity | Affiliation |
|---|---|---|
| Professor Fernando Jorge Monteiro. Eng. M.Sc. Ph.D. | Supervisor | Full Professor<br>University of Porto<br>Faculty of Engineering<br>Department of Materials and Metallurgy<br>Porto, Portugal |
| Professor Maria Helena Fernandes. Ph.D. | Advisor for Cell Culture Techniques | Full Professor<br>University of Porto<br>Faculty of Dentistry<br>Porto, Portugal |
| Professor Derek J Hansford. Eng. M.Sc. Ph.D. | Advisor for Microfabrication Technology | Associate Professor<br>The Ohio State University<br>Columbus (OH), USA |
| Professor Antonio de Aza. Ph.D. | Advisor for Ceramic Production | Ceramic and Glass Institute<br>CSIC – Consejo Superior de Investigaciones Científicas<br>Madrid, Spain |
| Professor Maria Pia Ferraz. Eng. Ph.D. | Advisor for Bacterial Culture Techniques | Associated Professor<br>Universidade Fernando Pessoa<br>Faculty of Health Sciences<br>Porto, Portugal |
| Professor Claudia Patricia García García. Ph.D | Advisor for thin films | Associated Professor<br>Universidad Nacional de Colombia<br>School of Sciences<br>Department of Physics<br>Medellín, Colombia |
| Professor Luis Felipe Restrepo Tamayo. DDS. Prosthodontics | Advisor for Clinical Dentistry | Instructor<br>University CES<br>Faculty of Dentistry<br>Medellín, Colombia |

| **From Professor Monteiro's Group. INEB – Instituto de Engenharia Biomédica. Universidade do Porto.** | | |
|---|---|---|
| Angela Carvalho. Eng. M.Sc. | Co-author in papers related with microfabrication | Former M.Sc Student |
| Liliana Grenho. Microbiologist. M.Sc. | Co-author in papers related with bacterial adhesion | Ph.D. Student |
| Rita Sousa | Pioneer in microfluidics in this group | M.Sc Student |

| **From Doctor Hansford's Group. The Ohio State University. Department of Biomedical Engineering.** | | |
|---|---|---|
| Nicholas Ferrell. Eng. Ph.D. | Co-author in publications related with microfabrication | Former Ph.D. Student |
| Daniel Gallego-Pérez. Eng. PhD. | Co-author in publications related with microfabrication | Former Ph.D. Student |
| Natalia Higuita-Castro. Eng. | First author in publications related with scaffolds of Portland Cement and Co-author in abstract related with bioactive particles of Portland Cement. | Ph.D. Student |

| **From Professor Antonio de Aza's Group. Ceramic and Glass Institute. Department of Ceramics. CSIC** | | |
|---|---|---|
| Professor Pilar Pena. Ph.D. | Advisor Ceramic Characterization | Professor Universidad Autónoma de Madrid. Madrid, Spain. |
| Marlin Magallanes. Ph.D. | XRD characterization | Former Ph.D Student |

| **From Laboratorio Nacional de Energia e Geologia. Portugal** | | |
|---|---|---|
| Jorge Ferreira. Ph.D. | Co-author in paper accepted in the Journal of Prosthetic Dentistry | Researcher |

| **From Universidad Pontificia Bolivariana** | | |
|---|---|---|
| Professor Santiago Arango. DDS. BMEs | Co-author in Review Paper | Former Specialist<br>Biomedical Engineering Research Group<br>Universidad Pontificia Bolivariana. |
| Professor Jorge Saldarriaga. M.Sc. | Co-author in Review Paper | Mechanical Engineering Department.<br>Medellín<br>Colombia |

| **From CES-LPH Research Group. University CES. Faculty of Dentistry** | | |
|---|---|---|
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| Alejandro Villegas. DDS. | Co-author in Poster presented in IADR | Former DDS |
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## SCIENTIFIC PUBLICATIONS AND COMMUNICATIONS

In the context of the present thesis the following publications and communications were done. They were grouped as papers published or submitted, (full papers, short papers and abstracts) and presentations in international scientific meetings.

### PAPERS PUBLISHED/ACCEPTED IN PEER REVIEWED JOURNALS (Ordered by Impact factor)

1) **Full Paper**. **PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, FERRELL N, FERNANDES MH, HANSFORD D, MONTEIRO FJ. Early spreading and propagation of human bone marrow stem cells on isotropic and anisotropic topographies of silica thin films produced via microstamping. MICROSCOPY AND MICROANALYSIS. 2010;16(6):670-6. (Impact factor 3.035).

2) **Full Paper**. **PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, MAGALLANES-PERDOMO M, FERNANDES MH, HANSFORD DJ, DE AZA AH, PENA P, MONTEIRO FJ. Isotropic micropatterned silica coatings on zirconia induce guided cell growth for dental implants. DENTAL MATERIALS. 2011;27(1):581-9. (Impact factor 2.882).

3) **Full Paper**. **PELÁEZ-VARGAS A**, DUSSÁN J, RESTREPO LF, PAUCAR CG, FERREIRA J, MONTEIRO FJ. The effect of slurry preparation methods on biaxial flexural strength of dental porcelain. JOURNAL OF PROSTHETIC DENTISTRY. 2011;105(5):308-14. (Impact factor 1.215).

4) **Review.** ARANGO S, **PELÁEZ-VARGAS A**, SALDARRIAGA-ESCOBAR J, MONTEIRO FJ, RESTREPO-TAMAYO LF. Ceramics for

dental restorations - An introduction. DYNA. 2010;163(77):7726-36. DOI: 49615099004.

5) **Short Paper**. **PELÁEZ-VARGAS A**, FERRELL N, FERNANDES MH, HANSFORD D, MONTEIRO FJ. Cellular alignment induction during early *in vitro* culture stages using micropatterned glass coatings produced by sol-gel process. KEY ENGINEERING MATERIALS. 2009;396-398:303-306.

6) **Short Paper**. **PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, FERRELL N, FERNANDES MH, HANSFORD DJ, MAGALLANES-PERDOMO M,. DE AZA A, PENA P, MONTEIRO FJ. Microfabricated $SiO_2$ Thin Films on Structural Dental Ceramics. BIOCERAMICS 22. 2009. Pag. 779-782.

7) **Full paper.** HIGUITA-CASTRO N, GALLEGO-PÉREZ D, **PELÁEZ-VARGAS A**, GARCÍA-QUIROZ F, POSADA OM, LÓPEZ L, SARASSA CA, AGUDELO-FLOREZ P, MONTEIRO FJ, LITSKY AS, HANSFORD DJ. Portland cement porous scaffolds for load-bearing bone tissue engineering applications. J Biomedical Materials Research – Part B. *Accepted. August 2011.*

**PAPERS OR CHAPTERS SUBMITTED, UNDER REVIEW, OR REJECTED IN PEER REVIEWED JOURNALS**

1) **PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, FERNANDES MH, HANSFORD DJ, MONTEIRO FJ. Effects of density of anisotropic micro-stamped silica thin films on guided bone tissue regeneration – *in vitro* study. *Under review in Dental Materials. July 2011.*

2) **Chapter in Book. PELÁEZ-VARGAS A,** GALLEGO-PÉREZ D, GÓMEZ DF, FERNANDES MH, HANSFORD D, MONTEIRO FJ. Propagation of human bone marrow stem cells for craniofacial applications. *Accepted and under editorial revision*: STEM CELLS AND CANCER STEM CELLS: Therapeutic Applications in Disease and Injury. Editor Professor Eric Hayat.

3) **Full paper. PELÁEZ-VARGAS A**, RAMÍREZ-FRANCO JH, FERNANDES MH, FERRAZ MP, MONTEIRO FJ. Bacterial adhesion and osteoblastic behavior on silica coatings optimized using statistical experimental design. *Rejected in Journal Chemistry of Materials and Colloids and Surfaces B: Biointerfaces. Some changes are necessaries to submit for a new journal.*

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**ABSTRACT PUBLISHED PEER REVISED JOURNALS (Ordered by impact factor)**

- **Microscopy and Microanalysis**

1) **Extended Abstract**. **PELÁEZ-VARGAS A**, FERRELL N, FERNANDES MH, HANSFORD D, MONTEIRO FJ. Cells spreading on Micro-fabricated Silica Thin film Coatings. MICROSCOPY AND MICROANALYSIS. 2009;15(Supp 3):77-78. doi: 10.1017/ S1431927609990845.

- **Journal of Dental Research**

2) **Abstract. PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, HIGUITA-CASTRO N, HANSFORD DJ, and MONTEIRO FJ. Zirconia

Microtextured Surfaces Using Soft Lithography. JOURNAL OF DENTAL RESEARCH. 2010;89(Spec Iss B):4004.

3) **Abstract**. **PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, HIGUITA-CASTRO N, HANSFORD DJ, and MONTEIRO FJ Microstamped Silica Films on Curved Zirconia Surfaces. JOURNAL OF DENTAL RESEARCH. 2010;89(Spec Iss B): 4005.

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8) **Abstract. PELÁEZ-VARGAS A**, CUARTAS JC, OSORIO L, MONTEIRO FJ. Microwave pre-drying effects on Feldspathic Porcelain

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11) **Abstract. PELÁEZ-VARGAS A**, DUSSÁN J, RESTREPO LF, MONTEIRO FJ. Structural effects of mixture techniques during the ceramic paste preparation. JOURNAL OF DENTAL RESEARCH. 2007;86(Spec Iss A):0380.

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- **Bone**

13) **Abstract. PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D D, FERNANDES MH, HANSFORD DJ and MONTEIRO FJ. Microstructured coatings to study the behavior of osteoblast-like cells on hard materials. BONE. 2011;48(Suppl 2):S105-S106.

14) **Abstract.** CARVALHO A, **PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, FERNANDES MH, HANSFORD DJ and MONTEIRO FJ. Proliferation of mesenchymal stem cells on stamped thin films modified with nanohydroxyapatite particles. BONE. 2011;48(Suppl 2):S106.

**OTHER PEER REVISED ABSTRACTS**

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16) **Abstract. PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, FERNANDES MH, HANSFORD DJ, MONTEIRO FJ. Micro-Structured Silica Coatings with HA Particles Using a PDMS Stamp. VV8.9. Proceedings MRS FALL Meeting SS5.29. Boston (MA). 2009.

17) **Abstract. PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, HIGUITA-CASTRO N, FERRELL N, HANSFORD D, MONTEIRO FJ. Silica coatings with Portland cement particles for dental implant surfaces based on sol-gel micromolding. Proceeding Biomedical Engineering Society (CD PS 10A-23). 2009

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# CONFERENCES/ CONGRESS / MEETINGS

- **International**

1) 3rd Joint Meeting of the European Calcified Tissue Society and International Bone and Mineral Society. May 7-11, 2011. Athens, Greece.
2) 50º Congreso de la Sociedad Española de Cerámica y Vidrio. Octobre 27-29, 2010. Madrid, Spain.
3) 88th General Session & Exhibition of the IADR. July 14-17, 2010. Barcelona, Spain.
4) Materials Research Society. Fall Meeting. November 30 - December 4. 2009. Boston (MA), USA.
5) Bioceramics 22. International Symposium on Ceramics in Medicine. October 26-29, 2009. Daegu, South Korea.
6) Annual Scientific Meeting of Biomedical Engineering Society. October 3-10, 2009. Pittsburgh (PA), USA.
7) 87th General Session & Exhibition of the IADR. April 1-4, 2009. Miami (Florida), USA.
8) Incomam '08. International Conference in Microscopy and Microanalysis and 43th Annual Meeting Portuguese Society for Microscopy. October 30-31, 2008. Porto, Portugal.
9) Bioceramics 21. International Symposium on Ceramics in Medicine. October 21-24, 2008. Búzios (RJ), Brazil.

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10) 86th General Session & Exhibition of the IADR. July 2-5, 2008. Toronto, Canada.

11) 42nd annual meeting of IADR-Continental European and Israeli Divisions. September 26-29, 2007. Thessaloniki, Greece.

12) 85th General Session & Exhibition of the IADR. March 21-24, 2007. New Orleans (LA), USA.

# ABSTRACT

Teeth lost due to factors such as caries and periodontitis remains as one of the most diffused health problems, as stated by the World Health Organization (WHO). The preventive and educational measures proved to be the most effective tools, independently of the socioeconomic factors of the population. Naturally other factors such as trauma and craniofacial abnormalities also affect the populations.

Since the introduction of dental implants in the eighties that Titanium became the gold standard for restorative therapy, bearing in mind the low biologic cost in comparison with alternative methods such as fixed prosthesis of several units. Its major success is due to initial osseointegration and implants high long-term survival rate.

In recent years a significant effort has been put in producing all ceramic dental implants. Although some commercial products consisting of all ceramic implants have gained some portion of the world implant market, there are still many question marks regarding biological, mechanical and economical aspects.

In the present work the following fundamental question was raised:
How would it be possible to improve the surface response of all ceramic dental implants? This is a very broad question, leading to the chance to exploit different scenarios, including guided tissue regeneration in periodontics, but also interesting for other craniofacial applications.
The main objective of this work was to modify the surface of dental ceramics through micropatterned glass coatings obtained using the synergy between sol-gel technology and microstamping. These surfaces were developed aiming at improving osteointegration and decrease bacterial adhesion.

To reach such aims the present work lead to:

1) The production of substrates using several dental ceramic materials (i.e. feldesphatic porcelain, In-Ceram discs, zirconia discs, and zirconia based framework cores by CAD/CAM).
2) The production of flat glass coatings by sol-gel method that were further modified by the inclusion of bioactive particles of nanohydroxyapatite aggregates or of modified Portland cement particles. These coatings were applied by dip coating or by spin coating techniques.
3) Obtaining masks by photolithography that were converted into silicone moulds, used in microstamping the hybrid sol produced by sol-gel, to obtain the micropatterned coated layers.
4) The evaluation of *in vitro* biocompatibility of the developed materials using several types of osteoblastic lineage cells (i.e. MG63, HOS y SaOs-2), gingival fibroblasts and bone marrow or dental pulp stem cells.
5) Exploratory *in vitro* study of bacterial adhesion to the obtained materials using oral cavity bacterial strains (i.e. *S. mutans*, *S. sobrinus* and *Porphyromona gingivalis*).

The present work had a multivariant design when considering different dental materials used as substrates, the introduced surface modifications, the evaluated cell types and the tested oral cavity bacterial strains. It should also be considered that a significant part of this work is the result of research carried our by the author while being a visiting student in several laboratories in Portugal, Spain and U.S.A.

The obtained results have distinct degrees of achievement. From the materials production point of view the following achievements were successfully obtained:

1) It was possible to obtain several dental ceramic materials to be used as substrates, including some commercialized materials. 2) Silica based micropatterned coatings were produced by sol-gel technology with different micropatterns (pillars and lines) with several inter-spacings (5, 10, 20 and 45 µm) in a range of scales between 500nm and 100 µm.

From the *in vitro* biologic function evaluation point of view of the several types of eucariotic human cells in different substrate/coating combinations, it was possible to show that, in the initial phases of adhesion and proliferation, all cell types presented directional alignment with the anisotropic coatings, this effect being reduced when inter-spacing was 45 µm.

This alignment was less evident for isotropic coatings. Besides, the effect of cell alignment control was not observed for culture times over 7 days of proliferation; however the available information allows to estimate that this effect is lost when cell multilayers are established, leading to a more natural organization. Finally increased cell activity and alkaline phosphatase releasing to the medium where observed after 7 days culture, when SaOs-2 cells were used.

The preliminary results of the motility of stem cells deriving from dental pulp on micropatterned surfaces, obtained by live-imaging microscopy have shown a high motility and directionality in the movements of cells lying on patterned surfaces when compared to similar flat surfaces.

The results on the decreasing bacterial adherence using quasi-static culture systems were not conclusive. Presumably this will require the use in the future of flux technologies with reactors or of microfluidics techniques. The synergy between micropatterning and sol-gel technology allows for the exploration of new surface modification alternatives for dental implants as

well as, to design new regeneration strategies for soft and hard tissues in craniofacial defects.

**RESUMO**

A perda de dentição devida a fatores como a cárie ou a periodontite continua a ser um dos problemas de saúde mais disseminados, de acordo com a Organização Mundial de Saúde (OMS). As medidas de prevenção e educação demonstraram ser as ferramentas mais efetivas, independentemente dos fatores socioeconómicos das populações. Sem dúvida que outros fatores como os traumas e as anomalias dentoalveolares afetam igualmente as populações.

Desde a introdução dos implantes dentários nos anos oitenta, que o titânio se converteu no padrão de ouro da terapia restaurativa, se tivermos em consideração o seu baixo custo biológico em comparação com outros procedimentos como a prótese fixa de várias unidades. Contribuem para o seu grande êxito a osteointegração inicial e a elevada sobrevivência dos implantes a longo prazo.

Nos últimos anos verificou-se um significativo esforço na produção de implantes dentários totalmente cerâmicos, baseados em zircónia estabilizada com ítria.

Embora alguns produtos comerciais consistindo de implantes totalmente cerâmicos tenham vindo a ganhar alguma cota do mercado mundial, existem muitas dúvidas ainda sem resposta em termos biológicos, mecânicos e económicos.

No presente trabalho foi colocada como pergunta fundamental a seguinte:

Como será possível melhorar a resposta da superfície dos implantes dentários totalmente cerâmicos? Esta é uma pergunta muito abrangente, que criou oportunidade para explorar diferentes cenários, incluindo a regeneração tecidular guiada em periodontia, mas também interessante para outras aplicações craniofaciais.

*Abstract*

O propósito deste trabalho foi o de modificar a superfície de materiais cerâmicos dentários a partir de revestimentos vítreos microtexturados, obtidos utilizando a sinergia entre a tecnologia sol-gel e a microestampagem. Estas superfícies foram desenvolvidas visando melhorar a osteointegração e diminuir a adesão bacteriana.

Para alcançar este objetivo, no presente trabalho levou-se a cabo:
A produção de substratos de diferentes materiais cerâmicos dentários (por exemplo, porcelanas feldespáticas, discos de In-Ceram, discos de alumina, discos de zircônia e coifas de zircônia usando CAD/CAM).
1) A produção de revestimentos vítreos lisos pelo método de sol-gel, modificados com inclusão de partículas bioativas, como microagregados de nanohidroxiapatite, ou partículas de cimento Portland modificado. Estes revestimentos foram aplicados por métodos de imersão/extração (dip coating) ou por rotação controlada (spin coating).
2) A obtenção de máscaras por fotolitografia, a partir das quais depois se obtiveram moldes de silicone, que foram utilizados na microestampagem do sol híbrido produzido por sol-gel, para produção de revestimentos microtexturados.
3) A avaliação da biocompatibilidade *in vitro* dos materiais desenvolvido, utilizando vários tipos de células de linhagem osteoblástica (por exemplo: MG63, HOS y SaOs-2), fibroblastos gengivais e células estaminais derivadas de medula óssea ou de polpa dentária.
4) Estudo exploratório *in vitro* sobre a aderência bacteriana aos materiais obtidos, usando estirpes bacterianas da cavidade oral (por exemplo: *S. mutans, S. sobrinus y Porphyromona gingivalis*).

O presente trabalho teve uma conceção multivariada quando se consideram os diferentes materiais dentários utilizados como substratos, as modificações de superfície realizadas, os tipos de células avaliados e as

extirpes bacterianas da cavidade oral testadas. Acresce que uma parte significativa deste trabalho resulta de trabalho realizado pelo autor enquanto estudante visitante de vários laboratórios em Portugal, em Espanha e nos Estados Unidos.

Os resultados obtidos têm graus distintos de estados de desenvolvimento. Do ponto de vista da produção de materiais obtiveram-se os seguintes sucessos:
1) Foi possível produzir substratos de diferentes materiais cerâmicos dentários incluindo alguns produtos comerciais. 2) Foram produzidos revestimentos de silíca microtexturados sobre diversos materiais dentários pela técnica de sol-gel, com diferentes padrões (pilares e linhas), vários espaçamentos entre padrões (5, 10, 20 e 45 μm) numa gama de escalas entre 500nm e 100 μm. 3) Foi possível demonstrar que se podem adicionar partículas bioativas do tipo hidroxiapatite ou cimento Portland aos revestimentos microtexturados.

Do ponto de vista da avaliação da função biológica *in vitro* dos vários tipos de células humanas eucarióticas nas distintas combinações de substratos e revestimentos, foi possível demonstrar que nas fases iniciais de adesão e proliferação, todas as células apresentaram um alinhamento direcional no caso dos revestimentos anisotrópicos, diminuindo este efeito para espaçamentos de 45 μm. Este efeito geral de alinhamento é menos percetível nos revestimentos isotrópicos. Para além disso, o efeito do controlo sobre o alinhamento celular não foi verificado para tempos de cultura superiores a 7 dias de proliferação; no entanto a informação disponível permite inferir que o efeito se perde com a geração de multicamadas celulares, produzindo-se uma organização mais natural. Finalmente observaram-se aumentos da atividade celular e da quantidade de fosfatase alcalina libertada para o meio de cultura após 7 dias de cultura, quando se usaram células SaOs-2.

Os resultados preliminares dos estudos de motilidade de células estaminais derivadas da polpa dentária sobre as superfícies microtexturadas, avaliadas com recurso a microscópio com seguimento automático acoplado a uma incubadora, mostraram uma maior mutilidade e direcionalidade no movimento das células sobre as superfícies texturadas em comparação com superfícies idênticas lisas.

Os resultados da diminuição da aderência bacteriana utilizando sistemas quasiestáticos de cultura não foram conclusivos. É previsível que seja necessário vir a utilizar no futuro técnicas que incluam tecnologia de fluxo com reatores ou tecnologia de microfluidos.

A sinergia entre a microestampagem e a tecnologia sol-gel possibilita a exploração de novas alternativas para a modificação de superfícies de implantes dentários, bem como para procurar novas estrategias de regeneração para os tecidos moles e duros para os defeitos craneofaciais.

**RESUMEN**

La pérdida dental por factores como caries y periodontitis, continúa siendo uno de los problemas de salud más diseminados según la Organización Mundial de la Salud (OMS). Las medidas de prevención y educación han demostrado que son la herramienta más efectiva, independiente de los factores socio-económicos de la población. Sin embargo, otros factores como el trauma y las anomalías dento-alveolares también afectan a la población.

Desde la introducción de los implantes dentales en los años ochenta, el titanio se ha convertido en el estándar de oro de la terapia de restauración, considerando su bajo costo biológico comparado con otros procedimientos como la prótesis fija de varias unidades. Pese a su éxito en la oseointegración inicial y a que la supervivencia de los implantes a largo plazo es elevada, en los últimos años se ha trabajado intensamente para producir implantes dentales totalmente cerámicos basados en circona estabilizada con itria. Aunque diversos productos comerciales de implantes totalmente cerámicos, tímidamente han ganado espacio en el mercado mundial, existen muchas preguntas por responder en terminos biológicos, mecánicos y económicos.

En el presente trabajo se definió como pregunta fundamental: Cómo puede mejorarse la respuesta de la superficie de implantes dentales totalmente cerámicos?, está es una pregunta amplia, que dió la posibilidad de explorar diferentes escenarios incluyendo la regeneración tisular guiada periodoncia así como para otras aplicaciones craneofaciales.

El propósito del presente trabajo fue modificar la superficie de cerámicas dentales a partir de recubrimientos vítreos micropatronados obtenidos utilizando la sinergia de la tecnología sol-gel y la micro-

estampación. Estas superficies fueron desarrolladas con la idea de mejorar la oseointegración y disminuir la adherencia de bacterias.

Para la obtención del objetivo descrito anteriormente, en el presente trabajo se realizó:

1) La producción de sustratos de diferentes cerámicas dentales (p.e: porcelanas feldespáticas, discos de In-Ceram alumina, discos de alumina, discos de circona y cofias de circona elaboradas por CAD/CAM).

2) La producción de recubrimientos vítreos lisos por el método sol-gel modificados con partículas bioactivas tales como micro-agregados de nano-hidroxiapatita o partículas de cemento Portland. Estos recubrimientos fueron aplicados por los métodos de inmersión/extracción o por rotación controlada.

3) La confección de máscaras por fotolitografía que después fueron convertidas a moldes de silicona, que se usaron en la micro-estampación del sol hibrido producido por sol-gel, para la fabricación de recubrimientos micro-patronados.

4) La evaluación de la biocompatibilidad *in vitro* de los materiales producidos, usando diferentes tipos de células del linaje osteoblástico (p.e: MG63, HOS y SaOs-2), fibroblastos gingivales y células estaminales derivadas de médula ósea o pulpa dental

5) Un estudio exploratorio de la adherencia bacteriana *in vitro* usando extirpes bacterianas de la cavidad oral (p.e: *S. mutans*, *S. sobrinus* y *Porphyromona gingivalis*) sobre los materiales producidos.

El presente trabajo tuvo un diseño multivariado, cuando se consideran los diferentes materiales dentales utilizados como sustratos, las modificaciones superficiales realizadas, los tipos celulares evaluados y las extirpes bacterianas de la cavidad oral utilizadas. Lo anterior, dado que una buena porción del trabajo fue realizado por el autor, en calidad de estudiante visitante en diferentes laboratorios de Portugal, España y Estados Unidos.

Los resultados obtenidos tienen diferentes grados de madurez: Desde el punto de vista de la producción de materiales se obtuvieron los siguientes logros: 1) Fue posible producir sustratos de diferentes cerámicas dentales incluyendo algunos productos comerciales 2) Fue posible producir recubrimientos silíceos micropatronados sobre diferentes materiales dentales por la técnica sol-gel, con diferentes formas (pilares y líneas), y a diferentes interespaciamientos (5, 10, 20 y 45 µm) con un rango de escala entre 500nm y 100 µm. 3) Fue posible demostrar que se pueden adicionar partículas bioactivas del tipo hidroxiapatita o cemento Portland a los recubrimientos vítreos micropatronados.

Desde el punto de vista de la evaluación de la función biológica *in vitro* de diferentes tipos de células humanas eucarióticas evaluadas en las diferentes combinaciones de sustratos y recubrimientos, se pudo demostrar que en las etapas tempranas de adhesión y proliferación, todas las células presentaron un alineamiento direccional en el caso de recubrimientos anisotrópicos, disminuyendo el efecto para los espaciamientos de 45 µm. Este efecto general de alineamiento es menos marcado en recubrimientos isotrópicos. Adicionalmente, el efecto del control sobre el alineamiento celular no fue posible cuantificarlo en tiempos mayores a 7 días de proliferación, pero con la información disponible se puede decir que el efecto del alineamiento de las celulas se pierde paulatinamente con el incremento de las multicapas celulares, tornándose en una organización más natural. Finalmente, se observaron aumentos en la actividad celular y la cantidad de fosfatasa alcalina liberada al medio después de 7 días de cultivo usando células SaOs-2.

Los resultados preliminares de los estudios de motilidad de células estaminales derivadas de la pulpa dental sobre superficies micropatronadas,

evaluadas utilizando un microscopio para seguimiento automático acoplado a una incubadora, mostraron una mayor motilidad y direccionalidad en el movimiento de las células sobre las superficies patronadas cuando se compara con una superficie lisa.

Los resultados de la disminución de la adherencia bacteriana utilizando sistemas quasi-estáticos de cultivo no son conclusivos. Es posible que sea necesario la utilización de técnicas que incluyan tecnología de flujo como reactores o tecnología de microfluídos.

La sinergia entre la fabricación por microestampación y la tecnología sol-gel, posibilita la exploración de nuevas alternativas para la modificación de superficies para implantes dentales, así como el diseño de nuevas estrategias de regeneración de tejidos blandos y duros para defectos craneofaciales.

# INTRODUCTION

Dento-alveolar trauma and congenital absences are the most important reasons to edentulism non-associated with bacteria. However, World Health Organization reports shown dental decay and periodontitis, two bacterial-origin diseases, as the most frequent diseases in humans. Ultimately, they lead to dental extraction procedures. Teeth loss compromises oclusal stability, masticatory efficiency and aesthetics. The prevention campaigns (education and fluorization), until now, are shown as the more cost/efficient methods to avoid this problem. However, in cases where the restoration is required, the titanium implant-based therapy appears as the "gold standard', considering that successful rates of ~95% after 5 years have been reported.

Branemark work described osseointegration as a biological phenomenon comprising direct contact between bone and Ti surfaces allowing for a new philosophy of therapy. Osseointegrated implants are good alternatives for replacing missing teeth but may present complications. Some implants do not integrate, nor survive enough, are affected by peri-implantitis or have poor aesthetics. Peri-implantitis is a chronic inflammation of the supporting tissues around the implant induced by bacterial colonization, facilitated by the implant/abutment gap, and by chemistry and surface roughness of the restorative components (Rimondini, Cerroni *et al.,* 2002; Klinge, Hultin *et al.,* 2005)

The outer surface of the peri-implant mucosa is lined by a stratified keratinized oral epithelium that is continuous with a junctional epithelium attached to the Ti surface by a basal lamina and by hemidesmosomes. The non-keratinized junctional epithelium is only a few cell layers thick in the apical portion and separated from the alveolar bone by collagen-rich connective tissue. This 3 to 4 mm biological barrier formed irrespective of the

original mucosal thickness, protects the osseointegration zone from factors released from the plaque and oral cavity (Klinge, Hultin *et al.,* 2005).

On transmucosal abutment of osseointegrated dental implants biofilms deposit and bacteria colonize. Like the gingival crevice around the natural tooth, the peri-implant mucosa covering the alveolar bone is closely adapted to the implant. In partially edentulous subjects, the developing microbiota around implants closely resembles the microflora of natural teeth (Klinge, Hultin *et al.,* 2005).

In addition to the dark-pigmented, gram-negative anaerobic rods, other bacteria are associated with peri-implant infections (*Bacteroides forsythus, Fusobact. nucleatum, Campylobacter, Peptostreptcc. micros and Prevotella intermedia*) (Tanner, Maiden *et al.,* 1997), and eventually *Staphylocc. spp, enterics, and Candida spp* (Leonhardt, Renvert *et al.,* 1999).

Although the ceramic crown covering the implant's abutment may be aesthetically optimal, the grayish color of Ti may appear through the peri-implant mucosa, thus impairing the entire aesthetic result (Tan and Saltzman, 2004). Also, due to soft peri-implant tissue recessions, the implant head might become visible over time.

Metals including Ti, may induce non-specific immunomodulation and auto-immunity. In immunologic *in vitro* tests, sensitization to Ti was observed (Lalor, Revell *et al.,* 1991). Such problems with Ti and ever growing expectations on aesthetics lead to increasing interest in all-ceramic implants.

Simulation studies indicated that Alumina abutments present a less favorable mechanical behavior when compared to Titanium-Zirconia abutments (Kohal, Att *et al.,* 2008), while other *in vitro* studies showed no

differences in fracture toughness, when ceramic abutments are used in comparison with titanium (Cho, Dong *et al.*, 2002).

Early trials for all-ceramics dental implant presented insufficient physical properties and were withdrawn. More recently various types of ceramics and novel processing methods were introduced for all-ceramic dental implants (Denry and Kelly, 2008).

New high-strength ceramics were developed, appearing to be tougher, less limited in their tensile strength, and less subject to time-dependent stress failure (Denry and Kelly, 2008).

In implant dentistry, abutments made of densely sintered yttria-stabilized zirconia (Y-TZP) have been introduced in implant-supported single-tooth crowns and are nowadays in use. These have tooth-matched color, good tissue compatibility, and lower plaque accumulation (Kohal and Klaus, 2004; Scarano, Piattelli *et al.*, 2004; Degidi, Artese *et al.*, 2006; Oliva, Oliva *et al.*, 2007). A prospective study evaluating *in vivo* zirconia abutments found survival rates of 100% after 4 years (Glauser, Sailer *et al.*, 2004).

Y-TZP implants have been used experimentally showing adequate but slow osseointegration (Akagawa, Hosokawa *et al.*, 1998; Kohal and Klaus, 2004). Current laboratory and clinical trials on Y-TZP implants, are encouraging, indicating that this new ceramic material could offer an optimal aesthetical restoration of missing teeth. Others ceramics like zirconia toughened alumina (ZTA) dense ceramics are also being developed, overcoming previous mechanical problems of alumina, aiming at competing with Y-TZP, and showing increased long term chemical and structural resistance and reliability (Hjerppe, Vallittu *et al.*, 2009).

The surface texture of dental implants affects the rate of osseointegration (Boyan , Lohmann *et al.,* 2001) and biomechanical fixation. Surface roughness is classified as "macro", "micro" and "nano" sized topologies.

The "macro" range, from millimeters to 10 µm, is directly related to implant geometry, with threaded screw and macroporous coatings helping the primary stability of the implants during early phase of implantation. However, high surface roughness may increase peri-implantitis risk, compared with moderate roughness (1-2 µm) within "micro" range (1-10 µm), maximizing bone/implant interlocking.

Surface profiles in the nano range play an important role in protein adsorption and osteoblasts adhesion and thus in osseointegration (Brett, Harle *et al.,* 2004) but no reproducible surface roughness is currently clinically available.

Glass coatings applied by sol-gel process and soft lithography are additive techniques where a high control of chemistry, roughness, thickness and textures are obtained (Curtis and Wilkinson, 1997; Pelaez-Vargas, Ferrell *et al.,* 2009b) producing controlled micropatterned textures.

Previous work from people directly or indirectly associated with this work showed:

- (Pelaez, Pareja *et al.,* 2005), have recently found in some preliminary tests, that glass coatings obtained by sol-gel on metallic surfaces present smoother surfaces when compared to TiN coatings, applied by PVD eventually allowing for reduced bacterial adhesion associated to peri-implantitis for the case of abutments. Increased roughness of

vitreous coatings containing HA particles appears to perform better in terms of osteoblast proliferation.

- Y-TZP and ZTA (De Aza, Chevalier *et al.,* 2002) may be adequate materials for implants and might be used as substrates for thin glass coatings.
- Glass Thin Films with bioactive particles enhance the *in vitro* and *in vivo* biocompatibility of the substrates (Duran, Conde *et al.,* 2004).
- HA nanoparticles can be produced with high control of composition. (Portuguese Patent Application 103528, International Patent Application PCT/PT2007/000031).
- Modified Portland Cement is a biocompatible materials for biomedical applications (Gallego, Higuita *et al.,* 2007).
- 3D and 2D micropatterns can be produced using microfabrication techniques such as a multilayer micromolding and photolithography (Gallego, Higuita *et al.,* 2007).

**GENERAL QUESTION**

How can we improve all-ceramics dental implants?

**OBJECTIVES**

The purpose of this thesis was to modify the surface of dental ceramics with micropatterned glass thin films obtained by soft lithography and sol-gel process technology, improving bioactivity and diminishing bacterial adherence.

To achieve the purpose described, the following specific objectives were defined in terms:

- **Dental Ceramic Substrates**
  1) To produce Feldspatic, Alumina and Zirconia dental ceramic substrates using different alternative ceramic processing.

- **Thin films**
  2) To evaluate compositional variations in the content of TEOS and MTES to develop glass sol-gel coatings with bioactive particles (hydroxyapatite or Portland cement) on dental ceramics.

- **Microfabrication**
  3) To study Soft Lithography as a methodology to produce micropatterned glass coatings on dental ceramics.
  4) To produce and compare sol-gel glass thin films containing nanometric HA particles or Portland cement.

- **Biological Behavior**
    5) To compare *in vitro* bacterial adherence of oral cavity native species on silica thin films.
    6) To compare biocompatibility using cells from primary cultures (bone, gingival and pulp) on thin films applied on dental ceramics.

**VARIABLES**

Variables explored in the present work are described in the Table 1.

**TABLE 1. VARIABLES EXPLORED IN DIFFERENT PHASES OF THE PRESENT WORK**

| Variable | Primary | Secondary | Comments |
|---|---|---|---|
| Ceramic Substrate | Glass<br>Zirconia (Y-TZP) | Alumina<br>Stainless Steel 316L<br>Titanium | |
| Ceramic Surfaces | Coated<br>Uncoated | | Uncoated: "as-prepared" |
| Bioactive Particles | Micro aggregates of Hydroxyapatite | Microparticles of Carbonated Portland Cement | |
| Silica Thin Films | Flat<br>Micropatterned | | Flat: applied by Dip and Spin Coating.<br>Microstamping. |
| Pattern's Geometry | Lines<br>Pillar | Multilevel resolution mask | |
| Pattern's Dimensions | Spacing | Spacing = 5, 10, 20 and 45 μm | Width = 5 μm<br>Heigth ~ 5 μm |
| Bioactive Particles | Micro- nano-HA | Portland Cement | |
| Human Cells | Primary cells<br>Osteoblast-like cells | | Primary: HBMSCs, HGF, PDMSCs.<br>Lines: MG63 and SaOs-2 |
| Bacterial Strains | *S. mutans*<br>*S. sobrinus* | *Porphyromona gingivalis* | |

Y-TZP. Yttria stabilized zirconia

nanoHA. Micro aggregates of nanoparticles of hydroxyapatite.

**THESIS STRUCTURE**

The present thesis is divided in two main sections: First section contains four chapters, where all the published papers were divided in state of art, ceramics substrates and silica coatings processing, biological behavior on microfabricated silica coatings and general conclusions. Second section, entitled Appendix contains some papers under review or edition, and abstracts published in Bone and Journal of Dental Research. The provisional versions of these manuscripts could suffer some alterations to fulfill requirements of editors, if these changes are made, these new versions will be added easily.

**Chapter 1.** ***"State of Art"*** was divided in two manuscripts:
This chapter has a short introduction for the sol-gel processing and soft lithography and their synergies. After, two manuscript accepted or published are presented

***First manuscript***, enentitled *"Propagation of human bone marrow stem cells for craniofacial applications"* was written to reply answer for a friendly invitation from Professor Dr. M.A. Hayat, editor of the book entitled "Stem cells and cancer stem cells" produced by Springer. This book chapter explores some clinical aspects of Cleft Palate and their challenges to repair bone defects, human bone marrow stem cells (HBMSCs) and other sources of Stem Cells (SC) to produce cell-based therapies and micro- and nanoscale control of the osteogenic cell microenvironment.

***Second manuscript***, entitled "*Ceramics for dental restorations – An introduction"*, was published in Dyna. This review paper presentes an historical evolution of dental ceramics, classification of dental ceramics and future perspectives.

**Chapter 2.** *"Ceramics substrates and silica coatings processing"* includes the ***third manuscript*** entitled *"The effect of slurry preparation methods on biaxial flexural strength of dental porcelain"*, evaluates the effects of two mixing techniques on the biaxial flexural strength and microstructure of dental porcelain. This paper published in The Journal of Prosthetic Dentistry.

In the present thesis other aspects of the processing of dental ceramics and silica thin films modified with bioactive particles were studied. A selection of these results is showed in the appendix section.

**Chapter 3.** ***"Biological behavior on microfabricated silica coatings"*** was divided in five manuscripts:

***Fourth manuscript***, entitled *"Cells spreading on micro-fabricated silica thin film coatings"*, describes the production of pillar-shape micropatterned silica coatings on glass substrates, and evaluates the adhesion of HBMSCs during an initial period (4h-24h). This extended abstract was published in a Special Issue of Microscopy and Microanalysis

***Fifth Manuscript***, entitled *"Early spreading and propagation of human bone marrow stem cells on isotropic and anisotropic topographies of silica thin films produced via microstamping"*, describes the production of isotropic and anisotropic microfabricated silica coatings obtained by sol-gel and to compare the biological behavior of HBMSCs on these surfaces at early stages of adhesion and propagation. This full paper was published in Microscopy and Microanalysis.

***Sixth Manuscript***, entitled *"Cellular alignment induction during early in vitro culture stages using micropatterned glass coatings produced by sol-gel process"*, compares the cell adhesion and early orientation of HBMSCs cultured on

micropatterned and on flat glass coatings produced by sol-gel. This paper was published in Key Engineering Materials.

***Seventh Manuscript***. *"Microfabricated $SiO_2$ thin films on structural dental ceramics"* describes the development of a technique to obtain microstructured $SiO_2$ coatings on 3Y-TZP based on soft lithography micropatterning and sol-gel processing. This paper was published in Bioceramics 22 – Proceedings of 22nd International Symposium on Ceramics in Medicine. In the sequence of this work was published the next paper.

***Eighth Manuscript***. *"Isotropic micropatterned silica coatings on zirconia induce guided cell growth for dental implants",* indicates how an alternative surface modification technique was developed to promote guided tissue regeneration on zirconia materials for application in dental implantology. This full paper was published in Dental Materials.

Other aspects of the biological behavior on coatings modified with bioactive particles (hydroxyapatite and Portland cement) were studied. A selection of these results is showed in the appendix section.

**Chapter 4**. ***"General Conclusions"*** contains conclusions and future perspectives divided in terms of substrates, flat and micropatterned silica thin films and biological behavior of HBMSCs, Human Gingival Fibroblast, Pulp-derived MSCs and osteoblast-like cells.

**Appendix.** This section is divided in aspects such as Dental Ceramic Substrates, Silica Thin Films and cellular behavior on materials developed, containing abstracts publisehed and draft of manuscript under revision.

# Chapter 1. STATE OF ART

**OVERVIEW**

This chapter is divided in a general section about basic concepts of where sol-gel and soft lithography, and two manuscripts:

**First manuscript**, entitled *"Propagation of human bone marrow stem cells for craniofacial applications"* was written to reply to answer for a friendly invitation from Professor Dr. M.A. Hayat, editor of the book entitled "Stem cells and cancer stem cells" produced by Springer. This book chapter explores some clinical aspects of Cleft Palate and their challenges to repair bone defects, human bone marrow stem cells (HBMSCs) and other sources of Stem Cells (SC) to produce cell-based therapies and micro- and nanoscale control of the osteogenic cell microenvironment.

**Second manuscript**, entitled "*Ceramics for dental restorations – An introduction",* was published in Dyna. This review paper presents an historical evolution of dental ceramics, classification of dental ceramics and future perspectives.

**BASIC CONCEPTS**

Biomaterials may be classified according to different approaches: Natural or synthetic materials is the simplest approach. Other classifications are based on aspects such as 1) *chemical structure,* where they are divided into metals and their alloys, polymers, ceramics, glasses, and composites or 2) *mechanical performance*, hard and soft materials, it is a common used classification as educative element in clinical dentistry. However, the new approaches can be based on regenerative medicine (i.e. micro-nanotechnology), where synthetic materials are having a restrictive area.

Considering this context, we have to think in futuristic classification where many synthetic materials will be no more than museum pieces, and all the biomaterials field could be dominated by natural biomaterials. Otherwise, we may look at the biomaterials field from a commercial point at view, in which the most realistic approaches include synergies between natural and synthetic materials to solve old questions. Any philosophical discussion about this competition (research vs. commercial) is far from the focus of the present work.

In different industrial applications, some hard materials can be improved using surface modifications, as described in the Table 2:

In general, these coatings are applied via thermal or chemical process with or without vacuum. Physical vapour deposition, chemical vapour deposition, and sol-gel processing.

Synergy is a word extensively used in the present work due that several mature materials and techniques were used. To find information about the first reference of works like zirconia, sol-gel, hydroxyapatite, soft

lithography, *S. mutans*, *S. sobrinus*, HBMSCs, Pulp-derived SC, gingival fibroblast was a hard task. Considering this problem, we decide use the year when this words were indexed in the MESH of in the National Library of Medicine (NLM), as shown in the Table 3.

**TABLE 2. HARD COATINGS FOR INDUSTRIAL AND MEDICAL APPLICATIONS**

| Coatings | Substrate | Applications |
|---|---|---|
| Ceramic | Ceramic | Bioactivation (Weng and Baptista, 1998; Griggs, Kishen *et al.*, 2003) |
| Glass | Ceramic | Bioactivation (Ferraris, Verne *et al.*, 2000) |
| Ceramic | Metallic and alloys | Bioactivation (Schrooten, Van Oosterwyck *et al.*, 1999; Ardlin, 2002; Balamurugan, Kannan *et al.*, 2003) |
| Ceramics | Polymeric | Protective (Ha, Gisep *et al.*, 1997; Diaz-Flores, Espinoza-Beltran *et al.*, 2001) |
| Polymeric | Metallic and alloys | Adhesion (Messer, Lockwood *et al.*, 2003) |
| Metallic and alloys | Glass | Wear (Briand, Weber *et al.*, 2004) |

**TABLE 3. INDEXING HISTORY IN PUBMED**

| Term | Year introduction | Definition |
|---|---|---|
| Zirconia | 1981 | Zirconium oxide. |
| | 2005 | Yttria-stabilized tetragonal zirconia polycrystalline. |
| | 2004 | In-Ceram Zirconia. |
| Orthosilicates for silica Sol-gel | 1984 | Tetraethyl orthosilicate (TEOS). |
| | n.f | Methyl trietoxy silane (MTES). |
| *S. mutans* | 1976 | A polysaccharide-producing species of streptococcus isolated from human dental plaque. |
| *S. sobrinus* | 1982 | A species of gram-positive, coccoid bacteria isolated from the human tooth surface. Strains have been shown to be cariogenic in experimental animals and may be associated with human dental caries. |
| *Porphyromona gingivalis* | 1994 | A species of gram-negative, anaerobic, rod-shaped bacteria originally classified within the bacteroides genus. This bacterium produces a cell-bound, oxygen-sensitive collagenase and is isolated from the human mouth. |
| Stem Cells | 1984 | Relatively undifferentiated cells that retain the ability to divide and proliferate throughout postnatal life to provide progenitor cells that can differentiate into specialized cells. |
| Fibroblast | 1964 | Connective tissue cells which secrete an extracellular matrix rich in collagen and other macromolecules. |
| Hydroxyapatite | 1975 | A group of compounds with the general formula $M_{10}(PO_4)_6(OH)_2$, where M is barium, strontium, or calcium. The compounds are the principal mineral in phosphorus deposits, biological tissue, human bones, and teeth. They are also used as an anticaking agent and polymer catalysts. |
| MEMS | 2009 | A class of devices combining electrical and mechanical components that have at least one of the dimensions in the micrometer range (between 1 micron and 1 millimeter). They include sensors, actuators, microducts and micropumps. |

n.f. Terms not found

Table 3 shows relevant information as some of the added terms may be considered truly consolidated, but, their respective year of indexation cannot support this statement.

Mature methods and materials were used in the present work, as above mentioned. Ceramic and glass substrates (i.e. glasses and zirconia), silica glasses (i.e. flat or structured coatings) sometimes modified with bioactive particles (i.e. micro-aggregates of nanohydroxyapatite or Portlant Cement Particles). Also, some polymers were essential to carry out many processes (i.e. PDMS, PPMA, PVA and PEG). However, for the reader a question might appear: What is new in the context of the present work?

An answer to this question can be established in information based in the number of references found in ISI WEB and Pubmed platforms. A search was carried out using as keywords grouped by:

- **Processing.** Soft Lithography, microstamping, sol-gel, PDMS.
- **Materials.** Silica.
- **Final product.** Thin film or coating.
- **Biology behavior.** Osteoblast, cells, bone.

More relevant results are summarized in Table 4. Based in the compiled information the following statement can be made: Microfabrication (using PDMS) to stamp ceramic and glass produced by sol-gel route to biomaterials applications is an unexplored way to modify biomaterials especially for dental implants based in the synergy of three solid areas of knowledge (microfabrication, sol-gel and dental biomaterials).

**TABLE 4. REFERENCES FOUND IN ISI WEB AND PUBMED**

| Keywords | ISI WEB platform (# references found) | PUBMED (# references found) |
|---|---|---|
| PDMS and sol-gel | 336 | 61 |
| PDMS and sol-gel and coating | 60 | 4 |
| PDMS and sol-gel and bone | 18 | 4 |
| Soft lithography and sol-gel | 91 | 10 |
| Soft lithography and sol-gel and biomaterial* | 1 (published in 2009) | 0 |
| PDMS and sol-gel and biomaterial* | 3 | 5 |
| Microfabrication | 3852 | 6719 |
| Microfabrication and sol-gel | 66 | 16 |
| Microfabrication and sol-gel and biomaterial* | 0 | 2 |

* Full keywords combination

**SOL-GEL AND SOFT LITHOGRAPHY**

Sol-gel is called as "*the wet chemistry*" to process ceramics, glass and other materials, but it can be referred to several chemical reactions occurring during the manufacturing of technological products. Under this name, several techniques of production of materials such as monolithics, powders, fibers, nanospheres, pigments and coatings have been developed for aerospatial, optical, electronic, refractory, automotive, polymers and sensors, medical industries (Nazeri, Trzaskoma-Paulette *et al.*, 1997; Zhang, Whatmore *et al.*, 1999; Zhong and Greenspan, 2000; Arkles, 2001; Chou and Cao, 2003).

Apparently, the first synthesis of silica was described by Ebelmen in 1844 but found the commercial application in the early sixties (Livage, 1997). Since its beginnings in the 40´s, the sol-gel processing helped to obtain a new generation of materials (ceramic and glasses). Considering the initial

precursors, the processing can be divided in aqueous solution of metal salts, metal alkoxide solutions or mixed organic and inorganic precursors (Dimitriev, Ivanova *et al.*, 2008). In the present work the alkoxides were used to produce a hybrid sol (TEOS/MTES). These precursors hydrolyze with the formation of partially hydrolyzed products products and subsenquently they undergo condensation with formation of an oxide network (Brinker, Ashley *et al.*, 1996; Guglielmi, 2010). The sol stage is used to produce thin films by spin coating, dip coating or stamping, the main process to produce the surface modification in the present work.

In general, these materials present high purity and homogeneity, its particle size distribution may be controlled at nano-scale level and require low temperatures to be prepared. In comparison with high temperature processes, these sol/gel materials and processes save energy, minimize evaporation losses and air pollution, in general not inducing reaction with containers. However, they are not free of disadvantages such as high cost of raw materials, large shrinkage during the processing, present residual hydroxyls, carbon and microporosity, long times for processing and are difficult to adapt to produce large pieces. For broader information the readers are invited to read more specialized reviews (Strawbridge, 1992; Brinker, Ashley *et al.*, 1996).

For optical applications can be used surface relief feature in or on thin films. Specific geometries are used to produce couplers, distributed feedback grafting, filters, lenses, beam splitters and mirrors. In their work (Fabes, Zelinski *et al.*, 1993) reported that (Roncone, Weller-Brophy *et al.*, 1991) used creative processes such as embossing grafting formed in a $SiO_2$-$TiO_2$ sol-gel waveguide using a master with 0.52 µm (interspacing) and 200 nm (height) master, or the waveguide was produced using an appropriate master of the sol that had been densified using laser light to write directly (laser 10.6 µm

$CO_2$ heats the surface of the $SiO_2$-$TiO_2$) or indirectly (laser Nd:YAG heats the substrate and produce the solidification).

For bioceramic applications (Hench, 1997) describe early that "*A common characteristic of glasses, glasses-ceramics and ceramics that bond to living tissues is the development of a bioactive hydroxyapatite layer in vivo at body temperature*". Also, this author proposed the following categories to materials with potential for biological or medical use: 1) Bioactive sol-gel coatings, 2) bioactive sol-gel glasses, 3) doped sol-gel matrices as biological and chemical sensors and 4) sol-gel matrices with entrapped living organisms.

To try and draw the history of photolithography is a hard task because it was born from lithography a slightly explored technique in the editorial industry. Later, photographers improved the process to achieve better printing results. However, this technique contributed later for an amazing development of the integrated circuit industry, and it became a main contributor for the information technology. Photolithography is also, essential to produce technology for sensors, microsensors, micromechanical systems (MEMS), microanalytical systems, micro-optical systems and integrated circuits.

In 2006, Mauro Ferrari wrote "*Less than twenty year ago photolithography and medicine were total strangers to one another …. And then, nucleid acid chips, microfluidics and microarrays entered the scene, and rapidly these strangers became indispensable partners in biomedicine*" (Ferrari, Desai *et al.*, 2006).

Basically, photolithography helps to create small structures in massive scale, but it is not always the best option for all applications. It requires expensive technology, with poor results for curved substrates, also is limited

to photosensitive materials. These limitations inspired two important papers introducing Soft Lithography (Xia and Whitesides, 1998).

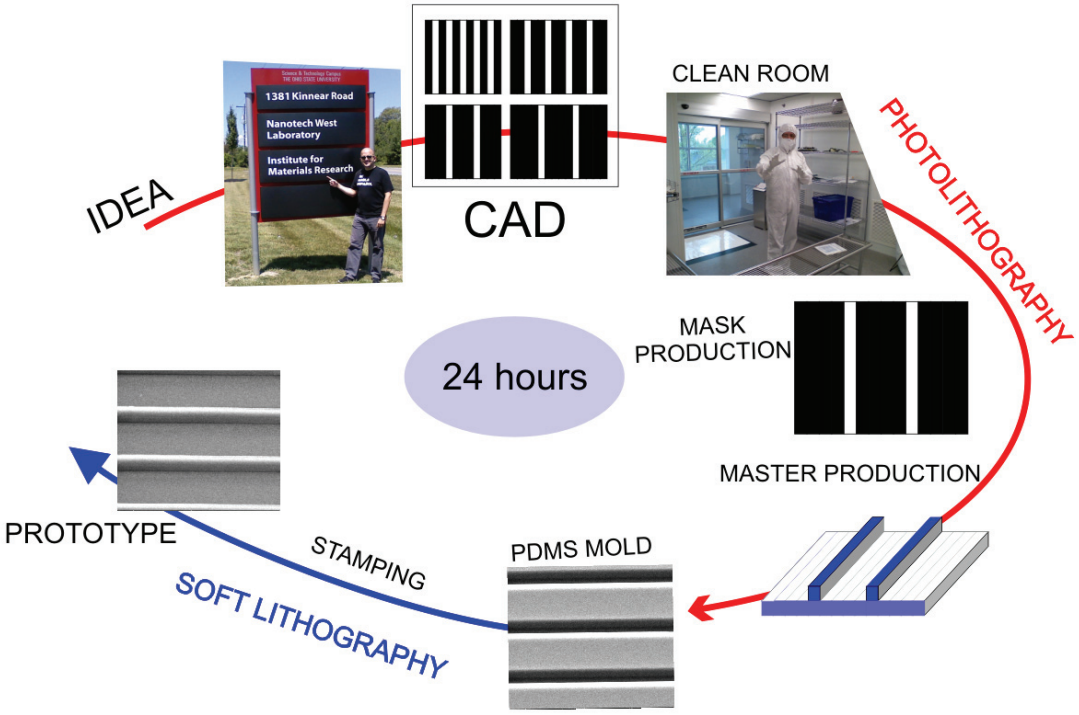



Figure 2. Rapid prototyping using Soft Lithography. Adapted from (Xia and Whitesides, 1998).

The soft lithography can be included between others techniques that basically are rapid prototyping process. Figure 2 showed a 24h flow from the idea to the final prototyping (Xia and Whitesides, 1998). To establish borders for the soft lithography is a hard task because several techniques such as microcontact printing (mCP), replica molding, microtransfer molding, micromolding in capillaries (MIMIC) and solvent-assisted micromolding (SAMIM), include the use of stamps or molds as key element to produce micro- nano patterns (Xia and Whitesides, 1998).

Elastomers have been used in most applications of stamping as poly-dimethylsiloxane (PDMS) from Dow Corning. Other groups have also used

other elastomers such as polyurethanes, polyimides, and cross-linked Novolac resin (a phenol formaldehyde polymer) (Xia and Whitesides, 1998). PDMS exhibits advantages such as being chemically inert, inexpensive, it presents good surface reproducibility, limited shrinkage, homogenity, it is isotropic, transparent, and easily modifiable by plasma. However, its main disadvantages are to swell in the presence of non-polar solvents such as toluene and hexane and forces as gravity, adhesion and capillary forces exert stress on the elastomeric features and cause them to collapse, generating defects in the produced pattern. Therefore, obtaining patterned surfaces with features smaller than 1 µm) is difficult (Xia and Whitesides, 1998).

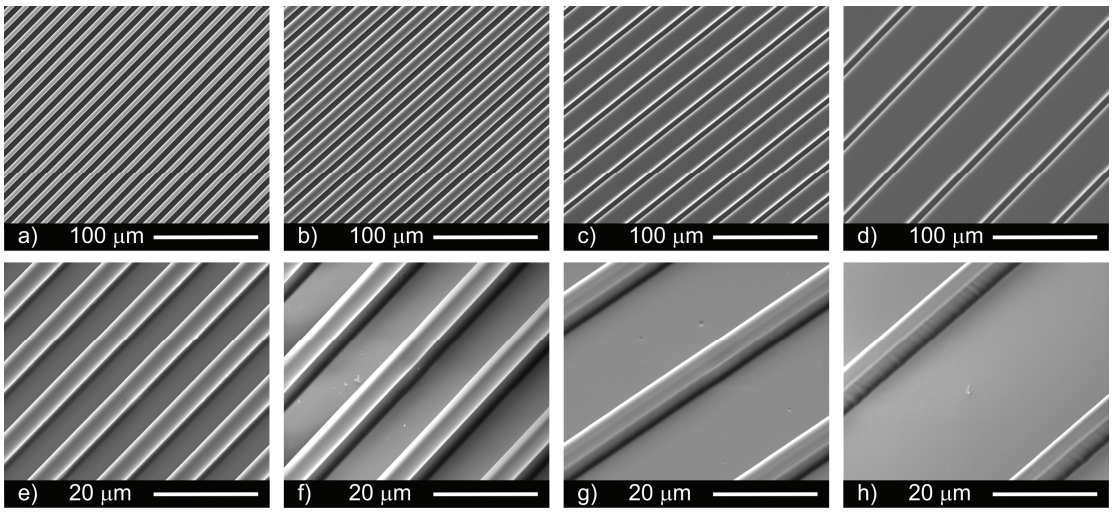



Figure 3. Anisotropic molds (a-d) and silica coatings (e-h).
The intespacings from left to right columns were 5 µm (a,e), 10 µm (b,f), 20 µm (c,g), and 45 µm (d,h), respectively.

Synergy between soft lithography and sol-gel was reported early to produce membranes and guide waveguides with size of 1 µm (Marzolin, Smith *et al.*, 1998). Figure 3 and 4 show anisotropic and isotropic PDMS molds and microstructured silica coatings produced by sol-gel. These shapes and dimension were selected for the present work, as that were expected to match the size of cells. Therefore they were used to evaluate the effects of these

surfaces on gingival fibroblast, HBMSCs, pulp-derived stem cells and osteoblast-like cells (MG63 and SaOs-2).

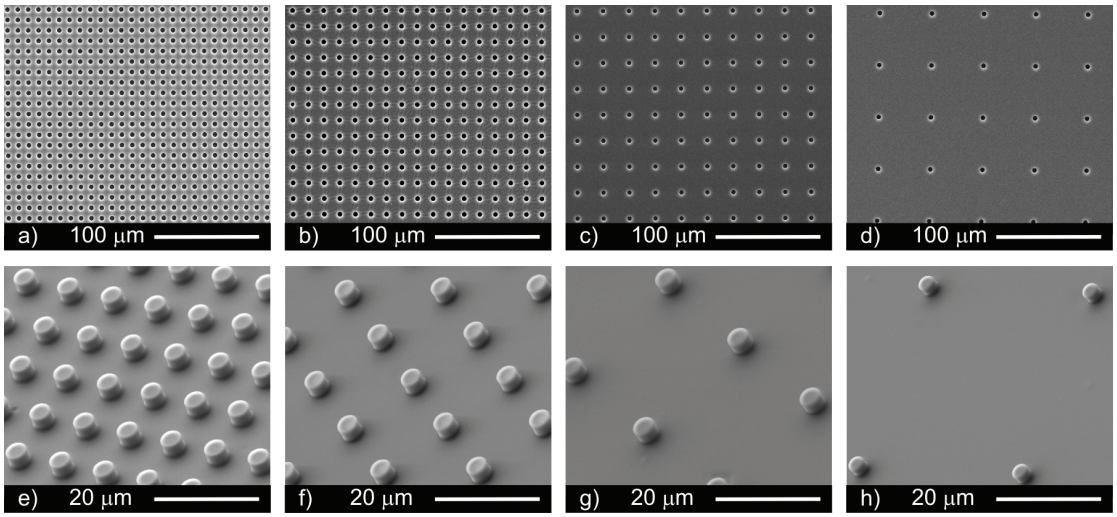



Figure 4. Isotropic molds (a-d) and silica coatings (e-h).

The interspacing from left to right columns were 5 μm (a,e), 10 μm (b,f), 20 μm (c,g), and 45 μm (d,h), respectively.

# Manuscript 1. PROPAGATION OF HUMAN BONE MARROW STEM CELLS FOR CRANIOFACIAL APPLICATIONS

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# PROPAGATION OF HUMAN BONE MARROW STEM CELLS FOR CRANIOFACIAL APPLICATIONS

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## ABSTRACT

Cleft palate is the second most frequent congenital defect in humans. Patients with this defect are treated by multi-disciplinary teams, which provide corrective procedures to improve aesthetic and function, as well as permanent psychosocial support. Frequently, these patients have corrective surgical procedures conducted to repair bone defects using different bone sources. Generally, modern surgical results are successful, although they can be improved in some aspects such as their reliability, especially for growing children. Novel combined approaches based in tissue engineering and Mesenchymal Stem Cells (MSCs) have been proposed. This chapter is mainly focused in the collection and *ex vivo* propagation of HBMSCs/ osteoprogenitors and their behavior in engineered microenvironments for craniofacial applications. Different aspects of isolation, propagation and osteoblastic phenotype expression are described. Also, the microfabrication

of micro-environments and their effects on molecular and cellular components are explained.

## INTRODUCTION

Reconstruction of significant portions of the craniofacial skeleton is commonly required as a consequence of trauma, tumors and craniofacial anomalies. Clefts of the lip and/or palate (CL±P) represent nearly one-half of all developmental defects of the face (Marazita and Mooney, 2004; Greene and Pisano, 2010). The epidemiology information related to these problems is not completely consolidated worldwide (Gundlach and Maus, 2006). The highest incidence in newborns with these challenging malformations is found in Asian and Amerindian populations (1 in 500), compared with European (1 in 1000) and African (1 in 2500) populations (Dixon, Marazita *et al.*, 2011). The proportion of patients with unilateral and bilateral clefts is 3:1 respectively (Gundlach and Maus, 2006).

Craniofacial anomalies are diagnosed at two different points in healthcare: prenatal or post-birth. An early diagnosis is possible using prenatal 3-D sonographic imaging that allows parents to accept and prepare for the future. On the other hand, a later diagnosis is made after the baby is born, which causes a more stressful situation for the parents due to the short period of time they have to assimilate the information and make decisions about the baby's health (Hodgkinson, Brown *et al.*, 2005).

Cleft lips/palates are not in the top of the morbidity/mortality rankings from the World Health Organization. However, they require treatment from a multidisciplinary team located in teaching hospitals around the world where plastic surgery procedures, specialized surgical orthodontic and dental treatment, speech therapy and psychosocial intervention are given in a sequential and progressive fashion, starting from intrauterine stages until adult age for the treatment of challenging patients (Association, 1993; Hodgkinson, Brown *et al.*, 2005).

In terms of dentofacial development and treatment, these patients present a challenge to the craniofacial team, due to the fact that they have to recover from distortions, displacements, and tissue deficiencies of nasal and maxillary structures as a result of the cleft, and have to receive a step-by-step treatment avoid disturbing the already altered growth of the craniofacial complex patients (Stenstrom and Oberg, 1961).

Figure 5 shows several patients with cleft lips and palates that required orthodontic treatment. They exhibited deficiencies in the alveolar bones and sagittal maxillary growths, a common characteristic. To re-establish a normal form and function in these patients, a correct diagnosis was required, followed by a prognosis and treatment. The prognoses were based on morphological factors as the size of the bone defect, the presence or absence of lateral incisor, the dental age of the patient, and the thickness of alveolar walls, among others. High rates of successful therapies may be obtained using

a careful surgical approach during the maxillary advancement and alveolar bone grafting (Figure 5a). However, these treatments are not free of undesirable results.

Figure 5b shows a patient with a marked resorption on the bucal wall of the alveolar bone that produced a tooth root exposed to oral cavity. The patient would need additional periodontal procedures to successfully cover this defect. In cases where a large bone defect is present (Figure 5c), a pre-surgical orthodontic therapy is required to reduce the defect which is wider in the basal portion than in the occlusal (dental) portion. The surgical team had to use different surgical techniques to fill the alveolar defect with bone. Finally, before and after images of a patient that underwent a successful maxillary distraction osteogenesis are presented (Figure 5d-e). After a latency period of 7 days and an active distraction phase of 20 days, a 12 mm of total advancement was obtained.

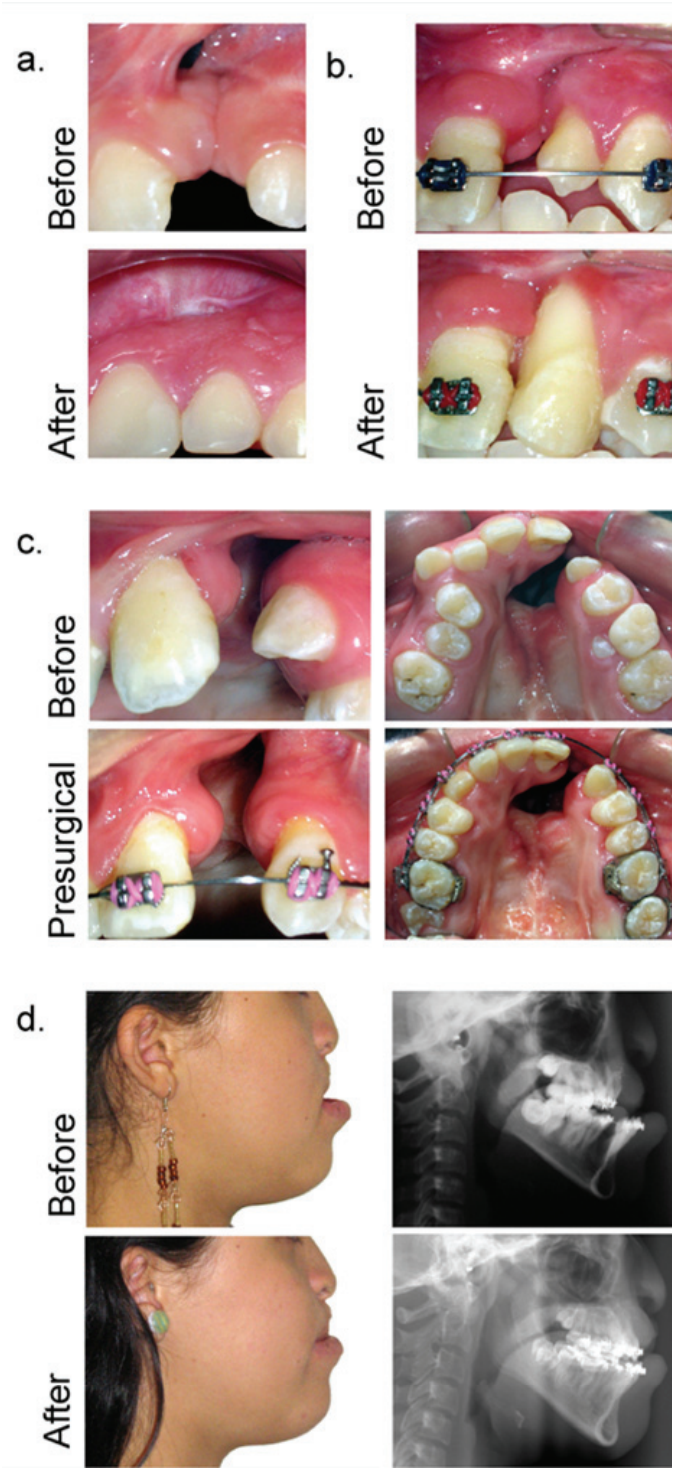



Figure 5. Treatment challenges in cleft lip and palate patients: a) successful and b) unsuccessful alveolar bone grafting; c) cleft alveolar bone at initial and presurgical stages, in which the bone grafting success is compromised by the extension of the alveolar defect; and d) before and after images of successful maxillary distraction osteogenesis, which can be enhanced by application of growth factors to speed the healing process of bone.

In the teen years, the patients have to undergo several procedures that involve bone grafting and bone distraction, as presented above. Current orthodontic and surgical techniques could be used with tissue engineering

strategies in a combined approach to modulate osteoblast growth and to achieve fast and predictable tissue regeneration. In this context, novel *in vivo* and *ex vivo* approaches such as bone grafting, growth factors, stem cell therapy, scaffolds, cell sheet engineering (Flores, Yashiro *et al.*, 2008; Ishikawa, Iwata *et al.*, 2009) or their combinations are available. Considering that *in vivo* approaches outside the focus of this chapter, the first step in basic and translational studies about *ex vivo* craniofacial bone regeneration would be the cell-culture techniques to obtain bone lineage cells. The present chapter is mainly focused in the collecting and propagation of human bone marrow stem cells (HBMSCs), and their early adhesion and proliferation on engineered microenvironments for craniofacial applications.

## 1. PROPAGATION OF HBMSCs FOR CRANIOFACIAL BONE REGENERATION

Bone, cartilage and soft tissues in the craniofacial complex are developed from intricate embryological processes governed by molecular mechanisms not completely discerned. It is accepted that these processes unique from others organs or systems. The development of maxilla and mandible and other structures, such as alveolar bone, dentin, pulp and periodontal ligament, have been associated with neural crest cells. Other structures such as axial and appendicular bones have been associated with mesoderm cells.

These origin differences imply the existence of progenitor cell niches in the bone marrow with specific properties (Akintoye, Lam *et al.,* 2006). Many preclinical trials of cell therapy for craniofacial complex have been generated using bone marrow as their cell source, from iliac crest aspirates or washed out of bone from orthopedic surgeries. Only recently has the source of cells for these types of orofacial interventions gained importance, after it was observed that bone grafts from the ilium were often rapidly resorbed when placed in maxillary or mandibular bone. Although it was not a frequent finding, more clinical information is needed to clarify if the progenitor niche may condition the cell activity in other host sites (Robey and Bianco, 2006).

Robey and Bianco (2006) reported that an estimated 96000 craniomaxillofacial bone grafts (6% of total bone grafts) are performed every year to regenerate bone lost due to trauma or disease. These procedures rely on autologous bone grafting, devitalized allogenic grafting (using bone from a bone bank) or natural and synthetic osteoconductive biomaterials. The main disadvantage of autologous bone grafting is that it is limited by the amount of bone that can be harvested and by donor site morbidity, while allogenic bone is often rapidly destroyed. Synthetic biomaterials have exhibited problems such as the resorption rate, differences in mechanical properties with the host bone, and they are not available in some world regions (costs or legal implications).

## a. Stem Cells

The terms Stem Cells (SCs), Adult Stem Cells (ASCs) and Mesenchymal Stem Cells (MSCs) were indexed in the National Library of Medicine (NLM) in 1984, 2007 and 2004, respectively. SCs may be divided in Hematopoietic Stem Cells (HSCs), cells that probably are the most explored for cell therapy, and MSCs, cells capable of differentiating into specialized cells to produce bone, cartilage, muscle, fat or other tissues.

The actual definition of MSCs in the NLM is "*relatively undifferentiated cells that retain the ability to divide and proliferate throughout postnatal life to provide progenitor cells that can differentiate into specialized cells*". However, many others definitions may be found in the literature. Considering that the present chapter is focused on the propagation of adult human bone marrow mesenchymal stem cells as a common phase in the *ex vivo* cell therapy for craniofacial applications, and that MSC culture is a rapidly expanding field of research (and thus difficult to define explicitly), readers are referred to more specialized reviews for more information (Pittenger, Mackay *et al.*, 1999; Bianco, Riminucci *et al.*, 2001).

As was well summarized previously (Robey and Bianco, 2006; Tuan, 2011), since 1960, Friedenstein and colleagues described the isolation and identification of a cell population from bone marrow, distinct from HSC, that was clonogenic and capable of protracted self-maintenance and differentiation into multiple mesenchymal cell lineages. This cell population

exhibited the ability to specialize into myelosupportive stroma, osteoblasts, adipocytes, chondrocytes, smooth muscle cells, and astrocytes, following *in vivo* transplantation (Tare, Babister *et al.,* 2008). Later, Caplan introduced the term MSCs (1991), and Pittenger and colleagues demonstrated the MSCs multi-lineage potential (Pittenger, Mackay *et al.,* 1999). In the specific case of bone, this hyper-hierarchical lineage starts from marrow stromal fibroblastic stem cells, and using appropriate stimulation they go through a sequence of osteoprogenitor, preosteoblast, osteoblast and osteocyte cells.

**b. Cell sources, explants and isolation**

The most common sources to isolate MSCs are perinatal tissues, such as placenta, umbilical cord and blood from the umbilical cord, and postnatal tissues, such as bone marrow, trabecular bone, alveolar bone, cartilage, hair follicles, fat, skin and dental pulp (Robey and Bianco, 2006). The marked differences of the harvest and separation methods for bone marrow found in the literature make it difficult to realistically estimate the frequency of MSCs. The information available provides an estimated frequency of the total nucleated cells is usually between 0.001 to 0.01%. However, this frequency shows age-dependence, from 1:10000 in a newborn to 1:1000000 in an 80-year-old subject (Bernardo, Locatelli *et al.,* 2009). Another aspect to analyze is the specific difference in terms of the donor site, as there is evidence that autologous bone grafting treatments, using different sources of bone cells,

show that the craniofacial donor site is usually more successful than other sites, which probably is associated with anatomic skeletal site-specific differences (Akintoye, Lam *et al.*, 2006). This supports the use of calvaria as a bone marrow donor site for grafting in craniofacial applications.

MSCs can be isolated with relative ease from BM and other tissues, and display fibroblast-like morphology. A brief technical description of two procedures to isolate MSCs is presented below.

One of the most convenient sources of human MSCs is from bone marrow aspirates, taken from the iliac crest under local anesthesia, but another source is a combination of bone tissue explants and bone marrow obtained from orthopedic corrective hip surgeries. In all cases, informed consent is required to collect and use these biological materials, which would otherwise be discarded.

Mechanical, chemical, or combined approaches to disrupt the extracellular matrix can be used to isolate MSCs from bone tissue bits. In the mechanical approach, the bone tissue is cut into small pieces using surgical blades, and then either suspended or plated. In bone marrow/bone chips suspensions, vortexing, shaking or flushing through needles, meshes or pipettes are also used, but these techniques require skills and experience, and they are not universally accepted, due to the cells susceptibility to shear-stress injury. Alternatively, the small bone chips or the bone pieces/cell suspension mixture are plated, preferably on tissue culture polystyrene (TCPS) culture

surfaces. The addition of adhesion-inductive biopolymers, serum or the use of micro/nano structured materials might improve cell adhesion, as they may give the possibility to create differential domains for cells. The close contact with, and stability on, the culture substratum is one of the most important conditions for establishing a cell culture using bone marrow, bone, or craniofacial tissues. This procedure, in general, is accepted as a reliable technique to obtain the desirable cell population (i.e. Figure 6a).

The enzymatic digestion of the bone chips, with a combination of trypsin and collagenase (chemical-based approach), to obtain a cell suspension should be avoided, as trypsin might damage the cell membrane surface. Also, regarding regulatory issues, characterization of trypsin and collagenase to fill the requirements of the regulatory agencies is a complex task, due to the difficulty in obtaining uniform compositions of these proteases. Still, the use of low concentrations of trypsin and collagenase may help to increase the efficiency of a mechanical process regarding the number of isolated precursor cells.

The number of sources for autologous MSCs for dentistry has increased, due to the hundreds of mandatory extractions (e.g., third molars and premolars for orthodontic reasons) done each year, as these tissues are routinely discarded. Many authors have demonstrated that MSCs derived from pulp of deciduous and permanent teeth, as well as from periodontal

ligament, might be isolated. These types of MSCs from different niches are heterogeneous and exhibit site-specific features, but in general are able to produce dentine, cement, and periodontal-like ligament structures (Robey and Bianco, 2006).

Upon cell adhesion and spreading, cell morphology is an initial criterion for assessing the success of the isolation procedure. However, in the case of heterogeneous populations, the classification based upon specific markers is desirable. It is generally accepted that a specific and unequivocal marker is not available, but that this may be satisfactorily solved using a non-specific multi-markers approach for MSCs-like cells. A reasonable characterization should be assumed if the analyzed MSCs cells are positive for STRO-1, CD73, CD146, and CD106, and negative for CD11b, CD45, CD34, CD31, and CD117, as recommended in (Tuan, 2011). However, for therapeutic applications, according to the International Society for Cellular Therapy, human MSCs must be plastic-adherent under *in vitro* culture conditions and express CD105, CD73, and CD90, while being negative for the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules. These expression profiles are most commonly measured using flow cytometry. In addition, cells must have the capacity for trilineage mesenchymal differentiation (osteoblasts, adipocytes and chondroblasts) under standard *in vitro* differentiating conditions (Dominici, Le Blanc *et al.*,

2006; Bernardo, Locatelli *et al.*, 2009). Another important characteristic is the chromosomal stability, in situations of extensive cell expansion. For instance, in alveolar bone cell cultures, excessive expansion has shown effects on the expression of the osteoblastic phenotype, namely a progressive decrease in matrix mineralization (Tomas, Carvalho *et al.*, 1997).

The contamination of established cultures with other cell types (e.g., hematopoietic or, in the case of gingival, epithelial cells) may be considerably reduced using simple techniques, based in different exposure times to a trypsin/EDTA solution following plating and expansion. However, whenever possible, high purification approaches should be used, relying on techniques involving magnetic sorting or fluorescence-active cell sorting (FACS), based on the presence of epitopes labeled with specific antibodies coated with magnetic or fluorescence beads, respectively. With FACS, it is possible to separate a heterogenic cell population using an automated system. This technique allows for multiple separations and for the collection of quantitative information on the percentage of each subpopulation of isolated cells.

### c. Cell cultures

Standard cell culture medium used in studies related with bone cell lineages include α-MEM (Minimal Essential Medium) and D-MEM (Dulbecco's Modified Eagle's Medium), supplemented with different

percentages of fetal bovine serum (FBS, 10% or 15% have been reported), antibiotics, antimycotics and ascorbic acid. Since 1997 the members of Fernandes' Group (co-author of the present chapter) have been studying the effects of different culture conditions on the behavior of bone-like cells using cell lines and primary cultures from different origins. A comparative study assessing the behavior of hBM osteoblastic cells, cultured in α-MEM or D-MEM, showed similar results regarding cell proliferation, however, cells cultured in α-MEM exhibited higher ALP levels and earlier formation of mineralized apposites (Coelho, Trigo Cabral *et al.*, 2000).

Serum is a complex mixture that includes proteins, growth factors, ions, lipids and hormones, which may promote or inhibit cell proliferation and differentiation. The choice of using FBS or Human Serum in the expansion of hMSCs may be addressed by considering different scenarios. First, if cells are expanded aiming at human applications, supplementation with autologous human serum is an unequivocal decision. Also, the use of FBS has some risk of zoonoses transmission. Accordingly, the U.S. Food and Drug Administration (FDA) recommendations/guidelines advise the elimination of the use of FBS to the greatest possible extent when generating cells for human use. Secondly, in basic studies addressing the proliferation/differentiation behavior of hMSCs, FBS (10%) appears to be the most used serum, as it is cheaper and easily available. However, in selected

situations, e.g., hematology departments in hospitals and schools of medicine, human serum is also used in basic studies, providing a more representative culture environment (Aldahmash, Haack-Sørensen *et al.*, 2011).

Also, there is a consensus in the literature regarding the relevance of testing different serum lots, as often differences can be found between batches and suppliers. The serum variations might be avoided if large stocks of previously tested FBS are acquired. This is a practice that increases the reliability of experimental results. Recently, Deorosan and Nauman (2011) evaluated the effects of the concentration of serum and glucose on the metabolic activity of murine MSCs, and concluded that the effects of the serum percentage (2%, 5% and 10%) were negligible, however a high correlated effect was found for cell viability and glucose concentrations (0.5 to 25 mM) (Deorosan and Nauman, 2011). In this context, serum-free strategies and "humanized" serum might be a desirable standard in cell-based therapies, as they provide comparable MSC proliferation and differentiation *in vitro*, while maintaining their bone production ability *in vivo*, with a cost reduction and less ethical implications (Even, Sandusky *et al.*, 2006).

In cell culture studies, the use of antibacterial and antimycotic agents is also a concern. Whenever possible, both should be avoided as they may affect cell physiology; also, the presence of these agents in the culture environment

may mask improper aseptic conditions. However, from a realistic point of view, considering the risk of losing irreplaceable cells, the standard use of antibacterials (e.g., Penicilin/Streptomycin, 100 IU/ml and 10mg/ml, respectively; Gentamicin, 10 µg/ml) and antimycotics (e.g., Amphotericin B, 0.25 µg/ml) is required. Other types of antibiotics, such as tetracyclines, are often used to take advantages of their Ca-binding and fluorescence properties under UV light, allowing for the quantification of the matrix mineralization. In this case, tetracyclines are added in high concentration (9 µg/ml) only few hours before the testing assay. As mentioned above, antibiotics might affect the cellular behavior, and regarding this, it has been reported that low concentrations (1 µg/ml) of doxycycline and minocycline (two tetracyclines) stimulate the proliferation of human osteoblastic cells (Gomes and Fernandes, 2007).

**d. Bone differentiation supplements**

The most frequent supplements for osteoblastic differentiation are ascorbic acid (AA, 50 µg/ml), dexamethasone (Dex, 10 nM) and β-glycerolphosphate (βGP, 10 mM) (Coelho and Fernandes, 2000). Ascorbic acid is a co-enzyme that stimulates the transcription, translation, and post-translational processing of collagen in connective tissue cells. Additionally, it has antioxidant features preventing the formation of reactive oxygen species. βGP is added to osteoblastic cell cultures to promote calcium phosphate

deposition by a mechanism that appears to be closely linked to the high ALP activity of bone cell cultures. It has been reported that the hydrolysis of βGP by ALP produces high levels of local phosphate ions, providing the chemical conditions for mineral deposition. Dex is a synthetic glucocorticoid added as an *in vitro* osteoinductive agent, due to its established positive effect on the proliferation and/or differentiation of osteoprogenitor cells in several osteoblast-like cell culture systems. Generally, *in vitro*, these compounds promote the growth and differentiation of osteoprogenitor cells, abbreviating the proliferation period of the developmental sequence in the expression of the osteoblastic phenotype (Amaral, Costa *et al.*, 2002).

Fernandes' group reported the proliferation/differentiation behavior of HBMSCs in the presence of different combinations of these supplements (AA, βGP, Dex, AA+ βGP, or AA+βGP+Dex). Results showed increased proliferation in the tested conditions, but only cultures performed in presence of βGP formed a mineralized extracellular matrix, and this event increased significantly in the presence of Dex (Coelho and Fernandes, 2000).

### e. Early cell adhesion, spreading and proliferation

Qualitative and semi-quantitative information on cell adhesion, spreading and proliferation may be obtained using light microscopy. However, more advanced imaging facilities such as Fluorescence Microscopy

and Confocal Laser Scanning Microscopy can provide more accurate data. After an appropriate fixation (1.5% formaldehyde, 15 min), permeabilization of the cell membrane (0.1% triton) and specific antibody immunofluorescence labeling, these techniques allow the visualization of multiple sub-cellular structures (e.g., nuclei, actin filaments, tubulin, and other cytoskeleton proteins). For bone researchers, live-imaging technologies are becoming more frequently available. Although the characterization of motility and invasion as properties of cancer cells are in the focus of many biologists, a lot of basic work is still required to learn more about the motility behavior of MSCs from craniofacial tissues.

**f. Assessing for matrix mineralization**

The formation of a mineralized extracellular matrix is the ultimate proof of the osteoblastic differentiation of MSCs. This event might be assessed in fixed cultures (1.5% glutaraldehyde, 10 - 15 min) by light, scanning and transmission electron microscopy. Histochemical staining of the fixed cultures provides a fast and simple method to assess the presence of alkaline phosphatase (ALP) and calcium phosphate deposits (Coelho and Fernandes, 2000). For ALP staining, the substrate (Na-α-naphtyl phosphate) is hydrolyzed by ALP at pH ~10, releasing phosphate and an aryl naptholamide. This compound couples immediately with a diazonium salt present in the incubation mixture (fast blue RR salt) forming an insoluble azo

dye at the theoretical sites of enzyme activity, identified by a brown to black stain (Figure 6b). Phosphate deposits are stained with the von Kossa assay: in the presence of silver nitrate solution, the phosphate present in the matrix forms silver phosphate, which is subsequently reduced to metallic silver (by UV radiation), shown by black deposits in the matrix (Figure 6c). Calcium deposits are assessed with the Alizarin red assay, in which Alizarin sodium, in aqueous solution, forms a chelate with calcium, causing a dense red precipitate (Figure 6d).

Scanning Electron Microscopy (SEM) provides more detailed information regarding cell behavior. Fixed cultures are dehydrated in a graded ethanol series (starting at 50% up to 100%) followed by critical point drying. Treatment with hexamethylsilazane, HMDS, might be an alternative, however, with some occupational risks. Sputter coating with a non-oxidizing metal like gold is required for imaging non-conducting samples in an SEM without other charge compensation mechanisms. SEM is based on the scanning of an electron beam over the sample, and the subsequent collection of electrons returning from the surface to form an image.

Field emission environmental (E) SEM devices, working at low-pressures (as opposed to the full vacuum of other SEMs) may help in the conservation of polymeric substrates and cellular structures (Figure 6e-g). Transmission

Electron Microscopy (TEM) is a powerful tool to study the ultrastructural cell response. Previously fixed cultures are post-fixed in potassium iron cyanide and osmium tetra-oxide aqueous solution (at 4°C) and, following dehydration through a graded series of ethanol solutions, samples are embedded in Epon and cut in ultra-thin sections. TEM uses an electron beam transmitted through the sample to image the cell ultrastructure. Cellular organelles, extracellular matrix and calcified nodules may be imaged in the higher resolution TEM (Figure 6h).

## g. Matrix mineralization in bone tissue applications

Osteoblast lineage cells are highly sensitive to the surrounding microenvironment, and matrix mineralization is affected in a variety of situations associated with bone repair/regeneration applications. Representative studies are summarized below.

In an early study, corrosion products from Co-Cr alloys were reported to affect the behavior of rat, rabbit and human bone marrow cells, namely cell viability, ALP activity, and the formation of a mineralized matrix (Tomas, Carvalho *et al.*, 1997). These effects were more pronounced in human cells, evidencing the importance of a representative cell system in the assessment of the bone cell response. Also, corrosion products from AISI 316 stainless steel (and the isolated Fe, Ni and Cr ions) showed deleterious dose- and time-dependent effects on ALP activity and matrix mineralization of rat and rabbit

BMSCs (Morais, Sousa *et al.*, 1998), as well as human alveolar bone cell cultures (Amaral, Costa *et al.*, 2002).

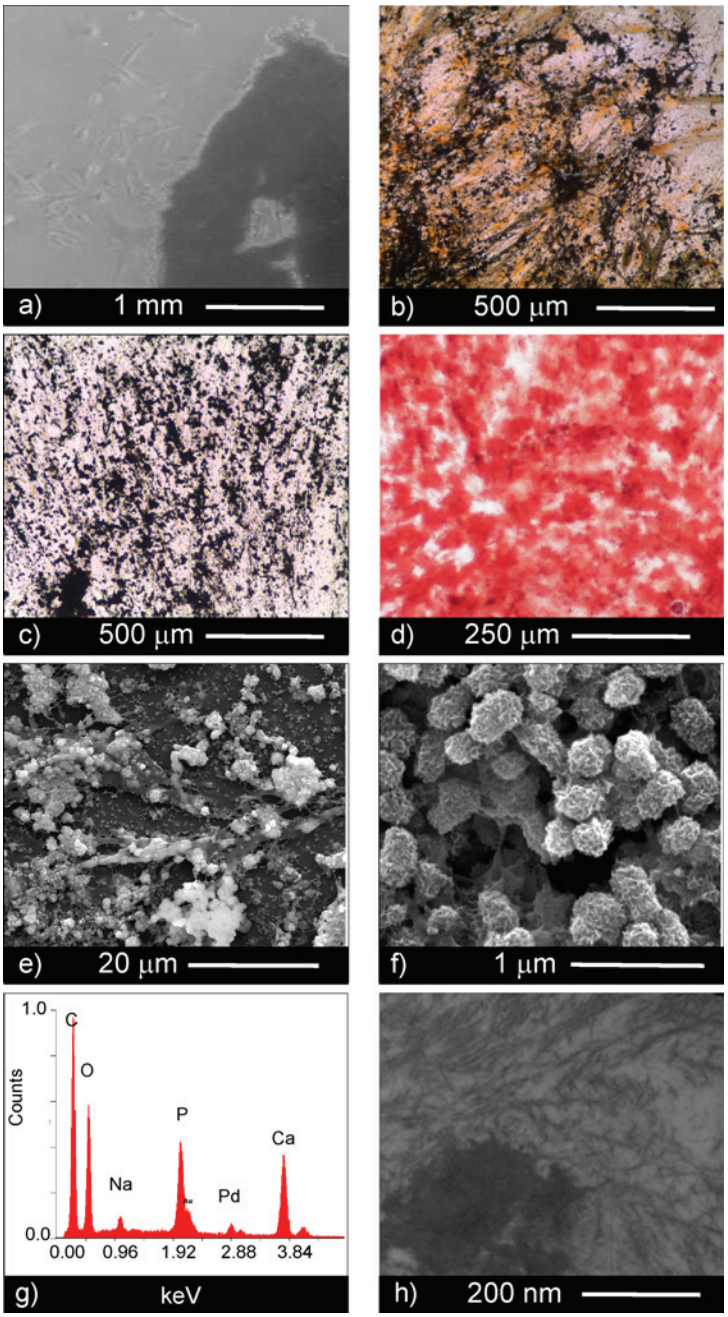



Figure 6. Behavior of MSC cultures performed in osteogenic conditions.

Light microscopy images showing the cell migration from a bone chip (a) and the histochemical staining of ALP (b) and phosphate (c) and calcium (d) deposits. SEM images showing a heavy cell-mediated mineralized matrix (e) and a high resolution image of the globular mineralized deposits (f) with the respective EDS spectrum displaying Ca and P peaks (g). TEM image exhibiting a mineralized deposit integrated in a fibrillar collagen network (h). a) 10-day primary culture; b) to h) 21-day first subculture.

Other biomaterials, theoretically more biocompatible, like hydroxyapatite or glass-reinforced hydroxyapatite, have been assessed in

human bone marrow osteoblastic cell cultures. Both materials are chemically close to the mineral phase of the bone tissue, and cells exhibited better adhesion, proliferation and differentiation in materials pre-immersed in culture medium compared to the "as prepared" materials (Teixeira, Monteiro *et al.*, 2007). Impairment of the cell behavior over the "as-prepared" materials is more significant in materials with high dissolution rates, and is associated with the ion release upon contact with the culture medium, resulting in a high ionic concentration in the cell microenvironment (Dias, Lopes *et al.*, 2005).

This observation shows the sensitivity of osteoblast cells to medium changes, and emphasizes the relevance of a proper material dissolution rate of phosphate bioceramics to achieve the desired cell response. On the other hand, the release of ionic species from hydroxyapatite-based materials might be explored to obtain a positive modulation of the osteoblastic cell response. For instance, Si-substituted hydroxyapatite coatings promote osteoblast proliferation and differentiation compared to hydroxyapatite coatings, due at least in part to the release of Si ions (Gomes, Botelho *et al.*, 2010).

Combined or synergistic effects of materials and relevant drugs might also be developed to induce the desired response of osteoblast lineage cells. For instance, the presence of doxycycline and minocycline (1 μg/ml) in glass-reinforced hydroxyapatite combined the positive effects of tetracyclines and those of the composite material on osteoblastic cells, compared to hydroxyapatite. Also, the local delivery of tetracyclines might combine the

antimicrobial activity of these compounds, to prevent implant related bone infection, with their positive effects on osteoblastic cells (Gomes, Santos *et al.*, 2008).

Osteoblastic lineage cell cultures are also a sensitive tool to analyze the effects of bioactive molecules, with therapeutic or toxicological interest. For example, it has been reported that chlorhexidine (CHX) and povidone–iodine, two main components of typical antiseptics solutions used in oral surgery, affected the behavior of alveolar bone cells, in a dose-dependent manner: low concentrations stimulated cell response, but levels of CHX ≥ 0.005 mg/ml or PI ≥ 0.5 mg/ml impaired cell proliferation and function (Cabral and Fernandes, 2007). In another study, dose-dependent effects were also noted in bone marrow cells exposed to nicotine, and results suggested a direct modulation of the osteoblast activity as a contributing factor to the overall effect of nicotine in the bone microenvironment around dental implants (Pereira, Carvalho *et al.*, 2008; Pereira, Carvalho *et al.*, 2009).

The studies mentioned above highlight the relevance of MSCs and their *in vitro* osteogenic differentiation as tools to assess bone cell response to materials and agents intended for bone tissue applications. Also, the high sensitivity of these cells to the modulation of the surrounding microenvironment demonstrates the potential to achieve desired and tailored bone cell responses in bone repair/regenerations strategies.

## 2. MICRO- AND NANOSCALE CONTROL OF THE OSTEOGENIC CELL MICROENVIRONMENT

Although scaffold-based bone tissue engineering approaches have shown great promise for the repair/regeneration of critical-sized defects, the varying degrees of success of such strategies suggests that the mechanisms involved in the regulation of bone regeneration, within a tissue engineering context, still remain to be fully elucidated (Petite, Viateau *et al.*, 2000; Hutmacher, Sittinger *et al.*, 2004). This regenerative/reparative process is known to be influenced in part by specific patient conditions (e.g., smokers, diabetics); however, it has also become increasingly clear that the regulation of the osteogenic cell microenvironment, via the scaffold's micro/nanoscale physicochemical properties, could play a significant role in the modulation of bone tissue formation.

Bone cell behavior is regulated via an integration of intrinsic and extrinsic factors. The intrinsic factors are for the most part contained in the genetic makeup of the cell. Extrinsic factors on the other hand are dictated by the cell microenvironment, which is comprised of soluble factors (Gerstenfeld, Cullinane *et al.*, 2003; Alford and Hankenson, 2006; Discher, Mooney *et al.*, 2009), extracellular matrix (ECM), and other cells (Aubin, 1999; Purpura, Aubin *et al.*, 2004; Alford and Hankenson, 2006; Jayakumar and Di Silvio, 2010). The osteogenic cell niche is therefore the source of a myriad of signals (e.g., biochemical, mechanical, electrical, structural, topographical) that act

independently, synergistically or antagonistically, with precise micro/nanoscale control, to govern bone cell behavior.

Micro- and nanoscale technologies, derived for the most part from the semiconductor industry, have been shown to offer unique capabilities to develop systems that can be used to control and/or probe many aspects of the osteogenic cellular microenvironment (Campbell, Miller *et al.,* 2005; Hosseinkhani, Hosseinkhani *et al.,* 2007; Mata, Kim *et al.,* 2009; Coutinho, Costa *et al.,* 2010; Pelaez-Vargas, Gallego-Perez *et al.,* 2010; Pelaez-Vargas, Gallego-Perez *et al.,* 2011a). Consequently, a better understanding of the interactions between bone cells and such micro-/nanoengineered environments could be crucial for the development of bone tissue engineering scaffolds with optimum micro/nanoscale properties (e.g., chemical, mechanical, structural).

Although a number of approaches have been studied to manipulate the microenvironment and influence bone cell behavior, strategies that aim at controlling the micro/nanoscale topography/architecture of biomaterials have been consistently shown to result in improved cellular responses (e.g., adhesion, propagation, proliferation, orientation, migration, differentiation, reactivity to hormones/growth factors) (Flemming, Murphy *et al.,* 1999; Martínez, Pradíes *et al.,* 2007).

For example, a growing amount of evidence suggests that micro-nanostructured biomaterials could play a significant role in improving bone tissue regeneration, both *in vitro* and *in vivo*, presumably by promoting favorable interactions with bone cells and/or different blood components (e.g. platelets, proteins, minerals) (Boyan , Lohmann *et al.*, 2001; Trisi, Lazzara *et al.*, 2003; Albrektsson and Wennerberg, 2004; Zinger, Zhao *et al.*, 2005; Shalabi, Gortemaker *et al.*, 2006; Christenson, Anseth *et al.*, 2007; Mendonca, Mendonca *et al.*, 2009). Besides the potential implications for the repair of critical size bone defects through scaffold-based tissue engineering, this concept is also actively being investigated for the development of optimum surfaces for endosseous implants. This section will focus on the effects of micro-/nanostructured biomaterials on bone tissue repair/regeneration.

The development of an optimum *in vivo* osteogenic cell niche, for the support of biomaterials-based bone repair/regeneration, depends in part on the initial interactions that occur between the biomaterial surface and different blood components. Micro/nanotextured biomaterial surfaces have been reported to enhance platelet adhesion and activation (Metroke, Parkhill *et al.*, 2001). This increased thrombogenic potential favors bone tissue regeneration via the formation of an osteoconductive and osteoinductive blood thrombus that coats the biomaterial surface (Body, 1996; Zhong and Greenspan, 2000).

Enhanced platelet adhesion and activation could possibly be attributed to the increase in surface area and protein adsorption, and/or to topographically-induced cytoskeletal rearrangement, which could lead to downstream intracellular signaling cascades that result in platelet aggregation and granule release. Other reports have proposed that the enhanced bone tissue responses, mediated by surface micro/nanotextures, could be due to the fact that the initial clot is stabilized by the topography via mechanical interlocking of the fibrin fibers (Grew, Ricci *et al.,* 2008; Mendonca, Mendonca *et al.,* 2009). In addition, micro/nanostructured biomaterials have also been shown to provide a greater number of nucleation sites for the precipitation of minerals (e.g. Ca and P) from the blood plasma, which results in the formation of an amorphous apatite layer that facilitates the interactions between the forming bone tissue and the biomaterial surface (Kokubo, Kim *et al.,* 2004; Wang, Hsu *et al.,* 2008).

Micro/nanostructured biomaterials could also lead to enhanced tissue regeneration by providing micro-environmental signals that directly influence bone cell responses. Previous studies have shown that the initial osteoblast-biomaterial interactions (i.e. adhesion, spreading and growth) could play an important role in leading to a long-term positive response at the bone-biomaterial interface. Wang and collaborators demonstrated that the adhesion force of osteoblasts to nanotextured titanium increases in direct proportion to the surface roughness (Kokubo, Kim *et al.,* 2004; Wang, Hsu *et al.,* 2008).

Similarly, Fewster and collaborators found that micron-scale (1-50 μm) pillars and pores on polymeric biomaterials (polystyrene and polyethylene terephthalate) could lead to improved osteoblast cell adhesion (Fewster, Coombs *et al.,* 1994). Nanostructured ceramic biomaterials (alumina) have also been shown to promote increased adsorption of ECM proteins (e.g., vitronectin) that result in superior osteoblast cell adhesion (Webster, Schadler *et al.,* 2001).

Additional studies, also confirmed that micro and nanotextured polymers, such as polylactic acid and polystyrene, could promote increased osteoblast cell adhesion in comparison to their untextured counterparts (Wang, Hsu *et al.,* 2008). In this study, both micro- and nanotextured surfaces resulted in similar levels of cell adhesion, while cell proliferation levels on textured and smooth surfaces were comparable. However, another study on the response of osteoblasts to topographies formed by silica nanoparticles reported lower levels of cell adhesion and proliferation on the nanoparticle patterns compared to flat surfaces (Kunzler, Huwiler *et al.,* 2007).

Interestingly, studies by other groups report significantly higher osteoblast cell proliferation on nanostructured titania surfaces compared to flat surfaces (Popat, Chatvanichkul *et al.,* 2007; Popat, Leoni *et al.,* 2007). Altogether, the differences found between these studies suggests that perhaps the extent of

bone cell responses depends on the specific chemistry of the biomaterial and/or the geometrical parameters (e.g. shape and size) of the nanotexture.

Surface micro/nanotextures are also capable of controlling cell morphology/spreading, which could have a direct impact on important biological processes such as cell alignment, migration, and functionality (Dike, Chen *et al.,* 1999; Flemming, Murphy *et al.,* 1999; Martínez, Pradíes *et al.,* 2007). Charest and collaborators showed that osteoblasts cultured on microgrooved polymide surfaces exhibited strong alignment/directionality with the topography (Charest, Bryant *et al.,* 2004). Recent studies by Peláez-Vargas and collaborators on micropatterned silica model implant surfaces (Figure 7) also show that these textures promote organized surface colonization by osteoprogenitor cells (Figure 8), which could potentially lead to guided bone tissue regeneration in the peri-implant region (Pelaez-Vargas, Gallego-Perez *et al.,* 2010; Pelaez-Vargas, Gallego-Perez *et al.,* 2011a). Other studies have reported edge-localized formation of focal adhesions by rat osteoblasts on micro/nanogrooved polystyrene surfaces (Matsuzaka, Walboomers *et al.,* 1999). Topographical modulation of the spatial distribution of focal adhesions has been postulated to lead to integrin-mediated intracellular signaling that could give rise to some of the observed cell behaviors on textured surfaces (Mendonca, Mendonca *et al.,* 2009).

Numerous studies have also reported that micro- and nanostructured biomaterials could lead to an enhanced osteogenic phenotype (Groessner-Schreiber and Tuan, 1992; Abron, Hopfensperger *et al.,* 2001; Popat, Chatvanichkul *et al.,* 2007; Popat, Leoni *et al.,* 2007), and in some cases, the combination of micro and nanostructures in the same system results in a cooperative synergy that ultimately results in improved cellular responses (Dalby, Riehle *et al.,* 2004). Figure 8a-b shows an example of a micropatterned silica implant model surface that also incorporates bioactive particles of nanophase hydroxyapatite (Fuentes, Toledano *et al.,* 2003). Studies performed with osteoprogenitor cells on semi-ordered and random polymethylmethacrylate nanocolumns have shown that such nanofeatures stimulate the differentiation towards an osteogenic phenotype, even in the absence of exogenous osteogenic factors in the culture medium (Dalby, Riehle *et al.,* 2004). Nanotextured titanium-based surfaces have also been shown to cause the upregulation of bone sialoprotein and osteopontin by rat osteoblasts (Novaes, Souza *et al.,* 2002).

Micro- and nanostructured surfaces have been shown to also influence the behavior of other cell types that could play an active role in the regulation of the osteogenic microenvironment. A recent study showed that osteoclasts are also sensitive to nanoscale surface features (Geblinger, Addadi *et al.,* 2010).

In this study the authors found that increased roughness led to enhanced resorptive activity by the osteoclasts.

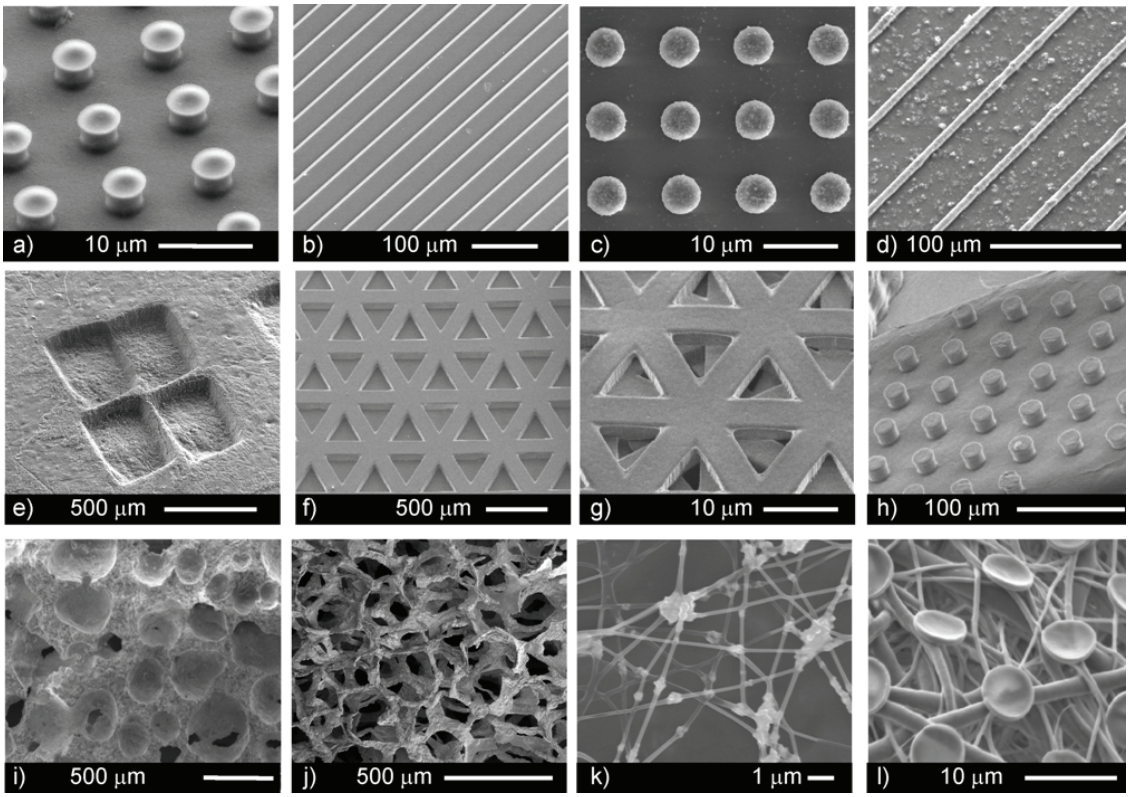



Figure 7. SEM images of different procedures to obtain micro- nanofabricated 2-D and 3-D surfaces.

Microstamped a) isotropic and b) anisotropic silica surfaces. Bioactive particles (HA) modified with c) isotropic and d) anisotropic patterns obtained using soft lithography. e) Laser-modified surface of a dental ceramic. Polymeric (i.e., polycaprolactone –PCL-) tissue engineering scaffolds fabricated via spin dewetting/ micromolding and thermal processing (Gallego-Pérez et al. 2010). f) Single layer and g) multilayer scaffolds. h) single layer scaffold with predefined surface topography. i) Portland cement and j) zirconia scaffolds fabricated using a combination of the foaming and particulate leaching methods. k) nanofibers obtained by electrospinning filled with ceramic particles and l) PCL scaffolding structure fabricated using a combination of electrospinning and soft lithography micromolding.

This has significant implications in the design of optimum biomaterial systems for bone repair/regeneration, as bone turnover could be a beneficial

process under controlled conditions; however, excessive bone resorption could be detrimental for the regenerative/reparative process.

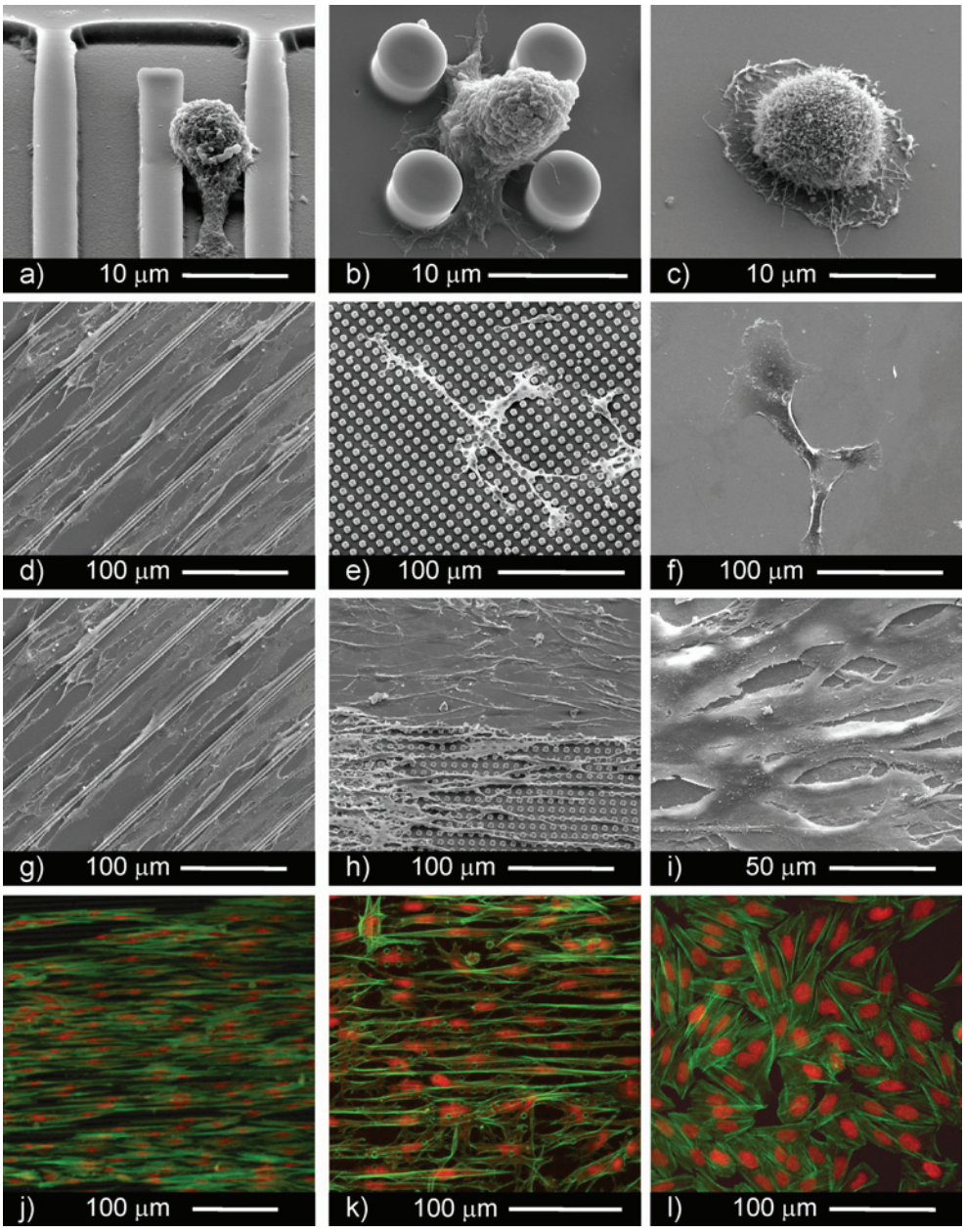



Figure 8. SEM and CLSM images of HBMSCs.

SEM images of the adhesion (4h, top row), early spreading (24h, second row) and proliferation (7 days, third row) of HBMSCs on line (left column) and pillar (middle column) micropatterned surfaces compared with flat (right column) surfaces. Images from Confocal Scanning Laser Microscopy showed HBMSCs aligned on line and pillar micropatterned surfaces after 14 days of culture. A random distribution can be observed on flat surfaces.

Similarly, previous studies conducted by Wojciak-Stothard and colleagues showed that murine macrophages also presented increased phagocytic activity when cultured on nanogrooved surfaces (Wojciak-Stothard, Curtis *et al.*, 1996).

The knowledge derived from the studies on the effects of micro/nanostructured biomaterials on bone cell behavior could then be used to properly engineer scaffolds that incorporate micro/nanoscale architectural, structural, and topographical cues that facilitate bone tissue repair and regeneration. Unlike traditional scaffold fabrication approaches (e.g., particulate leaching, foaming, sponge templating, phase separation, and freeze-drying) (Griggs, Kishen *et al.*, 2003; Navarro, Valle *et al.*, 2004; Kuo and Chung, 2005), which typically only provide partial control over the sub-millimeter scale structural properties (Figure 7i-j), micro/nanoscale technologies offer the possibility to assemble three-dimensional scaffolding structures with controlled physicochemical characteristics at the cellular and subcellular levels (Vozzi, Flaim *et al.*, 2003; Causa, Netti *et al.*, 2007; Tsang, Chen *et al.*, 2007; Gallego, Ferrell *et al.*, 2008; Melchels, Feijen *et al.*, 2010).

Figure 7 illustrates multiple examples where micro- nanoscale technologies have been used in the fabrication of polymeric or ceramic scaffolds with controlled features at the micro and nanoscale. Micro- and

nanotechnology-based approaches for the fabrication of tissue engineering scaffolds could potentially allow for the development of structures that can more closely resemble the complexity of the osteogenic cell microenvironment, thus favoring bone tissue formation.

## 3. FUTURE CONSIDERATIONS

Cleft palate anomalies and implantology are the craniofacial applications where more challenges remain to the implementation of tissue engineering-based solutions. Considering that cleft palate is the most frequent craniofacial congenital birth defect in humans, affecting 1 in 500 newborns, a large impact in public health may be expected if its therapies are improved. The tissue engineering technology is a promising approach to achieve bone regeneration, providing distinctive advantages for the control of cellular processes at micro- nano-scale. Mesenchymal stem/osteoprogenitor cells have shown improved *ex vivo* proliferation and differentiation under appropriate culture conditions. Combined techniques including stem cells, *ex vivo* cell cultures, and engineered surfaces may help to create favorable microenvironments for bone growth.

Micro-nanostructured surfaces present increased surface areas, which lead to enhanced protein adsorption and cell adhesion, and increased nucleation sites, which increase the deposition of minerals from plasma and induce the formation of osteoconductive/osteoinductive scaffolds. Also, these

surfaces may modulate aspects as cell alignment and directional motility, which could be used to promote guided cell regeneration.

The control of the microenvironment may improve the prognosis and reliability of grafting for bone defects. In addition, it is accepted that synthetic materials used as scaffolds are generally not suitable for growing patients; the autogenic materials have donor-site limitations in children, and allogenic materials have infection risks. Therefore, the consolidation of the knowledge derived from tissue engineering for pediatric therapy is an open arena. The combination of consolidated therapies with novel approaches may be a better manner to achieve new results in short time, compared with radical changes that normally have long periods of implementation.

Finally, these new advancements in the recovery of bone structure in the craniofacial field can be applied in severe craniofacial cases that had significant bone reduction due to inborn conditions. The described techniques could assure a healthy recovery from surgery in extended bone defects or speed the healing process after craniofacial distraction osteogenesis or other craniofacial or alveolar grafts.

## ACKNOWLEDGMENTS

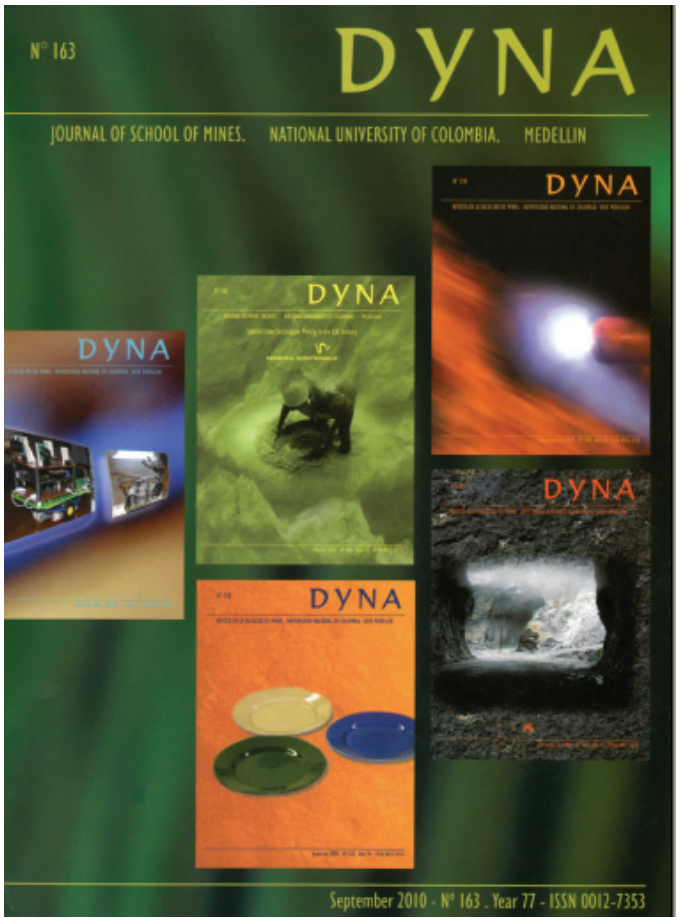
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## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

# Manuscript 2. CERAMICS FOR DENTAL RESTORATIONS – AN INTRODUCTION




CERAMICS FOR DENTAL RESTORATIONS – AN INTRODUCTION

UNA PERSPECTIVA DE LAS CERÁMICAS PARA RESTAURACIONES DENTALES

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# CERAMICS FOR DENTAL RESTORATIONS – AN INTRODUCTION

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## ABSTRACT

Dental ceramics are the preferred materials for oral restoration due to some characteristics, such as adequate esthetics, high fracture strength and chemical stability. Ceramics as dental materials have been used for more than 200 years; however, the most significant advances in mechanical properties and esthetics have been made in the last 40 years. Currently, dental professionals have a large amount of ceramic systems to choose from, all of them having small differences regarding their chemistry, processing temperatures, mechanical strength and clinical applications. These differences lead to classification systems that are difficult to understand by professionals outside the dental field. The aim of this work is to review the current dental ceramic systems and present them from a compositional perspective to assure the comprehension of these materials by professionals who belong to the Biomedical Engineering field. A great effort was made to avoid classification methods imposed by manufacturers and to obtain a quick

compilation of available information from the literature allowing the reader to have a general view of the state of the art on dental ceramics.

**Keywords**: Ceramic systems, feldspathic porcelain, leucite, zirconium oxide, glass-ceramics, mechanical and physical properties.

## INTRODUCTION

Dental ceramics are materials that are part of systems designed with the purpose of producing dental prostheses that in turn, are used to replace missing or damaged dental structures. The literature on this topic defines ceramics as inorganic, non-metallic materials made by man through the heating of raw minerals at high temperatures (Rosenblum and Schulman, 1997).

Ceramics and glasses are brittle, which means that they display high compressive strength but low tensile strength and may be fractured under very low strain (0.1% - 0.2%). As restorative materials, dental ceramics have disadvantages, mostly due to their inability to withstand functional forces that are present in the oral cavity, hence initially they found limited application in premolar and molar areas, although further development in these materials has enabled their use as posterior long-span fixed partial prosthetic restorations and structures over dental implants (Rizkalla and Jones, 2004). All dental ceramics display low fracture toughness when compared to other dental materials, such as metals (Rizkalla and Jones, 2004).

The main objective of this work is to review ceramic dental materials, including their most relevant physical and mechanical properties. A brief historical review, including the evolution of these materials over time, a

summary of different dental ceramic classifications, and the composition of dental ceramics will be presented.

## 1. HISTORICAL EVOLUTION OF DENTAL CERAMICS

Current dental ceramics are far from the early ceramics that started being used over 200 years ago. Early records of the first ceramics used as dental materials date back to 1774, when French apothecary Alexis Duchateau and Parisian dentist Nicholas Dubois de Chemant manufactured the first complete ceramic denture. There are reports of complete dentures being manufactured earlier by French dentist Pierre Fauchard, although these dentures were fabricated in a different class of ceramic, namely enamel (Jones, 1985).

The initial use of ceramic materials in dentistry was in the obtention of complete dentures. Early in the 19th century, Italian dentist Giuseppangelo Fonzi was capable of manufacturing individual ceramic teeth attached to a metallic substructure which, in turn, was attached to complete dentures. The restoration of individual ceramic teeth in the oral cavity was delayed until the late 1800s, when Logan constructed ceramic teeth fused to metallic posts so that these posts could function as an intra-radicular retention for the restoration (Jones, 1985; Kelly, Nishimura *et al.*, 1996).

The method to manufacture dental prostheses during the second half of the 20th century was through the fusion of ceramics and metallic structures that could function as a core. Metal-ceramic systems combine both the exceptional esthetic properties of ceramics and the extraordinary mechanical properties of metals (Rosenblum and Schulman, 1997). Some metals used as restorative materials in dentistry may constitute a problem for some patients. These problems may reveal themselves as allergies (Stejskal, Danersund *et al.*,

1999), gum staining (Arvidson and Wroblewski, 1978; Venclikova, Benada *et al.*, 2007), and release of metallic ions into the gingival tissue (Bumgardner and Lucas, 1995) and the gingival fluid (Mehulic, Prlic *et al.*, 2005). These drawbacks, as well as the search for more esthetic materials by patients and dentists, have stimulated research and development of metal-free ceramic systems.

During the last 40 years, research has focused on improving metal-free systems and developing superior materials regarding esthetics and clinical performance to offer patients several alternatives to restore missing or damaged teeth.

## 2. CLASSIFICATION OF DENTAL CERAMICS

Dental ceramics may be classified according to several parameters, such as their use, manufacturing temperature, ceramic system, composition, microstructure, and translucency. The classification used in this article will be based on composition. Due to the fact that this review will discuss some mechanical properties exhibited by dental ceramic materials, Table 5 summarizes the results of some mechanical tests published in the literature.

### a. Feldspathic porcelains

Before discussing this division of ceramics, it is important to keep in mind that most ceramics have two different phases: the glassy phase and the crystalline phase. The glassy phase is often responsible for the esthetic behavior, while the crystalline phase is associated with mechanical strength. However, the crystalline phase (leucite in feldspathic porcelains) has a similar diffraction index as the glassy matrix; hence it also contributes towards the translucency of the whole structure. Therefore, both mechanical and esthetic

properties are dependent on the ceramic composition (Martínez, Pradíes *et al.*, 2007).

Feldspathic porcelain is produced from the mixture of potassium feldspar, quartz, and kaolin. The latter improves plasticity and manipulation before the heating process. Other essential components are oxides of sodium, potassium, calcium, aluminum, and magnesium (employed to control ceramic's expansion coefficient and to try and match it with that of metals) (Weinstein, Weinstein *et al.*, 1962), zinc, iron, copper, titanium, nickel, manganese, and cobalt (as pigments) and tin, zirconium, and titanium (as opacifiers) (Anusavice, 1992). Feldspathic porcelain is highly brittle, hence it must be used as a veneering material in metal-ceramic and metal-free ceramic systems (Martínez, Pradíes *et al.*, 2007).

This type of ceramic is made of extremely fine powders to which de-ionized water or a special modeling liquid is added. A paste is thus formed to be tooth-shaped by a technician. Then, this paste must be taken to an oven set at a pre-established temperature and time depending on the ceramic being used. Currently, microprocessor-controlled ovens control all the thermal cycle steps and have optimized programs designed by manufacturers to process different types of ceramics (Knorpp, 1991). The heating process may be carried out either under vacuum or in air, however the process under vacuum reduces ceramic's porosity (Meyer, O'Brien *et al.*, 1976; Anusavice, 2004).

The objective is to bring the raw paste's particles together to form a paste that will have solidified upon cooling (Denry, 1996). During sintering, ceramics density will increase and this is associated to a volumetric contraction of 30 to 40% (Giordano, Pelletier *et al.*, 1995; Denry, 1996), although other researchers claim that this contraction is between20 and 25% (Conrad,

Seong *et al.*, 2007). During the paste formation process, it is essential to avoid bubbles or pores formation, since their presence will reduce the ceramics final mechanical strength, given that pores act as crack initiators (Tinschert, Zwez *et al.*, 2000). The sintering process considerably reduces porosity since this procedure involves, the melting/softening of the powder particle, causing reduction of surface energy, which renders pores to become spherical and volumetrically contracted, thus increasing densification (Rahaman, 2003).

Some methods to strengthen porcelain and avoid crack initiation and/or propagation have been reported by different authors. The most common methods are ionic exchange (chemical exchange of smaller sodium ions for larger potassium ions to create a compressive stress on the surface) (Dunn, Levy *et al.*, 1977; Anusavice and Hojjatie, 1991; Anusavice, 1992; Seha Mirkelam, Pamuk *et al.*, 2005; Rosa, Yoshimura *et al.*, 2009), thermal tempering (forced cooling of ceramic in air) (Anusavice, Gray *et al.*, 1991; Anusavice, 1992; Asaoka, Kuwayama *et al.*, 1992) or crack-propagation interruption by means of induction of residual compressive stresses on the surface (air-injection tempering) (DeHoff, Anusavice *et al.*, 1996).

### b. Leucite-reinforced feldspathic porcelain

Feldspar may form leucite, which is a potassium aluminum silicate mineral with a high thermal expansion coefficient (Anusavice, 2004). Leucite may be present in feldspathic porcelain in two forms: Firstly, the formation of this mineral through the incongruent melting of potasic feldspar (incongruent melting is a process by which a material is melted to form a liquid and a different crystalline material (Anusavice, 2004). Secondly, the aggregate of leucite to ceramics as synthetic powder (Cesar, Yoshimura *et al.*, 2005). Leucite-reinforced ceramics may be used to manufacture metal-ceramic restorations. The large difference between thermal expansion coefficient of

metals and that of ceramics is one of the problems encountered when manufacturing metal-ceramic restorations (Nielsen and Tuccillo, 1972; Bertolotti, 1980) as well as the difference in thermal expansion coefficients between layers of ceramic (Fairhurst, Anusavice *et al.*, 1981) when cooling to room temperature. This imbalance creates residual stress between both materials that may trigger failure at the interface (Vasquez, Ozcan *et al.*, 2008). Hence, a layer to layer compatibility involving ceramics and underlying metal is required (Nielsen and Tuccillo, 1972).

Also, leucite content in ceramics is associated to an increase in crack propagation strength (Cesar, Yoshimura *et al.*, 2005). A mechanism by which this increase is produced is the difference between leucite's thermal expansion coefficient (22 to 25 X $10^{-6}$/°C) and that of the glassy matrix (8 x $10^{-6}$/°C), resulting in the formation of tangent compressive stresses in the glass around leucite crystals, that undergo a phase transformation from cubic to tetragonal upon cooling (Denry, 1996). Sodium content has an effect on the tetragonal phase stability at high temperatures and lowers the thermal expansion coefficient, but it also decreases the ceramic's overall strength (Sheu, O'Brien *et al.*, 1994). Another mechanism of strength increase is explained by microcrack formation within and around leucite crystals when cooling. Both mechanisms are essential to prevent crack formation and propagation in the ceramic (Denry, Mackert *et al.*, 1996). Some researchers have suggested that ceramics reinforced with cubic leucite at room temperature display less flexural strength and less fracture strength than tetragonal-leucite ceramics (Denry, Mackert *et al.*, 1996).

Pinto *et al* assessed the effect that pH has on leucite-reinforced ceramics. These authors concluded that an acidic pH (3.5) produces a reduction in overall strength in leucite-reinforced ceramics (Pinto, Cesar *et al.*, 2008).

### c. Aluminous ceramics

Aluminous ceramics were first developed by MacLean and Hughes to manufacture prosthetic dental crowns (McLean, 2001). The principle of this type of ceramics is that a dispersion of high strength and high elastic modulus crystals exists within the glassy matrix to strengthen and harden the ceramic (McLean, 2001).

Alumina has the property of strengthening feldspathic porcelain, thus making it more fracture resistant. This fact may be observed when comparing aluminous ceramics strength to that of feldspathic porcelain (Sherrill and O'Brien, 1974) and when comparing hardness and strength values of aluminous ceramics with those of other ceramic systems (Al-Shehri, 2002). Alumina particle size has been proposed as a factor that improves particles agglomeration and some mechanical properties, such as fracture strength, of the partially-sintered alumina matrix (Chaiyabutr, Giordano *et al.*, 2009).

The presence of alumina decreases glasses' inherent brittleness and the risk of de-vitrification, which is a process whereby ceramics become opaque and brittle as a result of their crystallization, due to loss of the glassy structure (Alvarez-Fernández, Peña-López *et al.*, 2003).

### d. Glass-infiltrated composites

Currently, there is a ceramic system marketed as In-Ceram (Vita-Zahnfabrik, H Rauter GMBH & Co.KG, Bad Säckingen, Germany) that includes glass-infiltrated porous sintered alumina (Sorensen, Kang *et al.*, 1998). This system, developed by Tyszblat, is comprised of an aluminous core that is further infiltrated with molten glass to fill-in the voids left by aluminous particles in order to obtain a restoration that exhibits better physical properties when compared to traditional ceramics (see Table 5) and very similar to those of natural teeth (Tyszblat, 1988).

**TABLE 5. REPORTED VALUES FOR CERAMIC SYSTEMS, ENAMEL AND DENTIN SUBJECTED TO SEVERAL MECHANICAL TESTS**

| Material | 3-point bending [MPa] | Hardness [GPa] | Fracture Toughness |
|---|---|---|---|
| Feldspathic porcelain | 98 – 101 (Oilo, 1988) | 712.9 - 763.9 kg/mm2 6.98 - 7.48 GPa (Pelaez-Vargas, Saldarriaga *et al.*, 2007) | 1.0 - 1.49 $MPa/m^{0.5}$ (Drummond, King *et al.*, 2000) |
| Alumina-reinforced ceramic | 116 – 117 (Oilo, 1988) | 3.7 – 5.2 (Rizkalla and Jones, 2004) | 1.95 $MN/m^{3/2}$ (Al-Shehri, 2002) |
| High-alumina content ceramic | 155 (Oilo, 1988) | 5 - 10.8 (Rizkalla and Jones, 2004) | 4.58 $MN/m^{3/2}$ (Al-Shehri, 2002) |
| Glass-ceramic | 239.2 (Oilo, 1988) | 3.4 – 4.1 (Rizkalla and Jones, 2004) | 1.28 $MN/m^{3/2}$ (Al-Shehri, 2002) |
| Leucite-reinforced glass-ceramic | 78.37 - 133.56 (Drummond, King *et al.*, 2000) | 6.53 (Al-Shehri, 2002) | 1.26 $MN/m^{3/2}$ (Al-Shehri, 2002) |
| Lithium disilicate-reinforced glass-ceramic | na | 5.6 - 6.2 (Gonzaga, Cesar *et al.*, 2008) | na |
| Zirconium oxide ceramic | 800 – 1590 (Ardlin, 2002) | 1188-1274 kg/mm2 11.64 GPa (Vleugels, Yuan *et al.*, 2002) | 4.6 – 6.2 $Mpa\ m^{1/2}$ (Vleugels, Yuan *et al.*, 2002) |
| Dental enamel | na | 343 kg/mm2 3.36 GPa (Craig and Peyton, 1958) | 0.70 – 1.16 $KC(M\ N/m^3/2)$ (Hassan, Caputo *et al.*, 1981) |
| Dentin | 137.9 – 220.63 (Rasmussen and Patchin, 1984) | 64.75 - 73.75 kg/mm2 0.63 – 0.72 Gpa (Fuentes, Toledano *et al.*, 2003) | 3.08 $MN\ (m)^{-1.5}$ (El Mowafy and Watts, 1986) |

na. Data not available

### e. Alumina Polycrystals

High-alumina ceramics contain a minimum of 95% pure alumina (aluminum oxide, $Al_20_3$) (McLean, 2001). Andersson and Oden (Andersson and Oden, 1993) described the use of a high-purity (99.9%), densely-sintered alumina ceramic for the manufacturing of dental restorations (Andersson and Oden, 1993). Alumina displays a 15% to 20% contraction that must be compensated to manufacture such restorations. These investigators compensated its high contraction by using enlarged models of teeth and restoration manufacturing using a technique known as copy-milling to obtain a properly-sized finished restoration. The amount of expansion necessary to be applied to the models may be calculated from the sintering of compacted powder (Andersson and Oden, 1993). Copy-milling is better described by Andersson elsewhere (Andersson, Bergman *et al.*, 1989).

### f. Glass-ceramics

According to McLean (McLean, 2001), the first works on glass-ceramics were performed by MacCulloch, but his work did not receive much attention. Further investigations by Grossman and Adair (Grossman, 1973; Adair, 1982) concluded with the development of a tetrasilicic fluormica-containing ceramic system. According to both investigators, its composition is as follows: 45-70% $SiO_2$, 8-20% MgO, 8-15% $MgF_2$, 5-35% $R_2O$+RO, where $R_2O$ has a range between 5-25% and is composed of at least one of the following oxides: 0-20% $K_2O$, 0-23% $Rb_2O$, and 0-25% $Cs_2O$ to improve translucency and RO, which has a range between 0-20%, and is composed of at least one of the following oxides: SrO, BaO, and CdO. Additional components may account for up to 10% of $Sb_2O_5$ and/or up to 5% of traditional glassy colorants (Grossman, 1973; Adair, 1982).

The thermal treatment known as ceramming (Anusavice, 2004) is composed of two processes: glass is heated up to a temperature where nuclei form (750° - 850°C) and this temperature is kept for a period of time ranging from one to six hours so that crystalline nuclei form in the glass (process known as nucleation). Then, the temperature is risen to the crystallization point (1000°-1150°C) and this temperature is maintained for a period ranging from one to six hours until the desired level of glazing is obtained (process known as crystallization) (Stookey, 1956; Grossman, 1973; Adair, 1982). Developers of this type of ceramic system claim that fracture strength and hardness are better than those of traditional ceramic systems (Grossman, 1973; Adair, 1982), although other authors have shown that after the ceramming process a surface layer that decreases its strength and esthetic characteristics forms on this ceramic (Denry and Rosenstiel, 1993). However, some researchers claim that fractures in this type of ceramic have an internal origin, but no explanations are provided as to what particular factor causes fractures to appear in restorations manufactured with this material (Thompson, Anusavice *et al.*, 1994). However, there is an investigation that suggests that this ceramic's strength and hardness may be improved by performing a chemical shift on the surface layer (addition of lithium fluoride) (Denry and Rosenstiel, 1993).

## g. Leucite-reinforced glass-ceramics

Pressed glass-ceramics are materials containing high amounts of leucite crystals (35% by volume) (Anusavice, 2004). The basic component of this ceramic is feldspathic porcelain consisting of 63% $SiO_2$, 19% $Al_2O_3$, 11% $K_2O$, 4% $Na_2O$, and traces of other oxides. Leucite crystals are added to the aluminum oxide (Probster, Geis-Gerstorfer *et al.*, 1997; El-Mowafy and Brochu, 2002). This material is manufactured using a process known as heat-pressing, which is performed in an investment mold. This mold is filled with the

plasticized ceramic, thus avoiding the sintering process and the subsequent pore formation (Sorensen, Choi *et al.*, 1998). This ceramic undergoes dispersion strengthening through the guided crystallization of leucite. Dispersion strengthening is a process by which the dispersed phase of a different material (such as alumina, leucite, zirconia, etc.) is used to stop crack propagation, since these crystalline phases are more difficult to penetrate by cracks (Kelly, 1990; Anusavice, 2004). Leucite crystals are incorporated during ceramming, hence performing this process again is unnecessary when inducing crystal growth (Probster, Geis-Gerstorfer *et al.*, 1997).

The method of dual ionic exchange has been proposed in the literature to increase the resistance of this ceramic to separation, as long as potassium and sodium ions are available in the ceramic for the exchange. Long-term chemical effects on the ceramic should be further studied (Fischer and Marx, 2001).

### h. Lithium disilicate-reinforced glass-ceramics

This material is also a heat-pressed glass-ceramic with a 60% content of lithium disilicate crystals, which form an intertwined structure after being pressed, hence fracture strength is increased (Sorensen, Choi *et al.*, 1998; Mansour, Al-Omiri *et al.*, 2008). $Li_2O$ and $SiO_2$ aid in the crystallization of the required phase of lithium disilicate, BaO and $Cs_2O$ stabilize residual glass and $Al_2O_3$ and $B_2O_3$ render this ceramic chemically durable (Brodkin, Panzera *et al.*, 2002). The crystallization process is comprised of two steps: nucleation (one hour at 645°C) and crystal growth (4 h at 850°C) (Brodkin, Panzera *et al.*, 2002).

## i. Zirconium oxide ceramics

Since Garvin et al. (Garvie, Hannink *et al.*, 1975) published their work entitled "Ceramic steel?, zirconia has been considered a tough ceramic. Zirconia occurs as a natural mineral called baddeleyite. This mineral contains 80% to 90% zirconium oxide. The major impurities are usually $TiO_2$, $SiO_2$, and $Fe_2O_3$. This oxide exists in three different crystal structures: monoclinic at room temperature, tetragonal at ~1200°C, and cubic at 2370°C. A phase transformation from tetragonal to monoclinic occurs during cooling. This change produces a volumetric expansion ranging from 3% to 4%, which in turn causes crack formation within the material. Oxides such as CaO, MgO, $Y_2O_3$, and $CeO_2$ are added to pure zirconia to stabilize it in multiphase materials known as Partially-Stabilized Zirconia (PSZ). These materials basically consist of cubic zirconia as the major phase and precipitates of monoclinic and tetragonal as the minor phase at room temperature (Covacci, Bruzzese *et al.*, 1999). At room temperature, the tetragonal phase is in metastable state, since transformation from tetragonal to monoclinic may be induced by external factors, such as pressure or temperature (Guazzato, Albakry *et al.*, 2004; Pilathadka, Vahalova *et al.*, 2007). The resulting compressive stress from the volumetric expansion developed at the vicinity of a crack and the energy needed to carry out such phase transformation, provide this ceramic with high fracture strength (Deville, Chevalier *et al.*, 2003; Guazzato, Albakry *et al.*, 2004).

This process is known as transformation toughening (Covacci, Bruzzese *et al.*, 1999; Guazzato, Albakry *et al.*, 2004; Zivko-Babic, Carek *et al.*, 2005; Pilathadka, Vahalova *et al.*, 2007). It may generate a process known as ceramic aging, which is the spontaneous transformation from metastable tetragonal zirconia to monoclinic zirconia (Covacci, Bruzzese *et al.*, 1999), therefore a decrease in mechanical properties may occur in the ceramic over time (Deville, Chevalier *et al.*, 2003). An investigation carried out by Ardlin

demonstrated that strength was not affected by aging, although the ceramic's crystalline structure and surface were, indeed, affected (Ardlin, 2002).

As mentioned above, pure zirconia may be stabilized with $Y_2O_3$. Yttrium oxide is used to retain the tetragonal phase at room temperature as much as possible after sintering and avoid the monoclinic phase, since the accumulation of the latter is associated with a decrease in strength, toughness and density (Piconi, Burger *et al.*, 1998). The resulting stabilized ceramic is known as Tetragonal Zirconia Polycrystals (TZP) and when it is doped with 2-3% mol of yttrium oxide, this material is known as Yttrium Tetragonal Zirconia Polycrystals (3Y-TZP) (Covacci, Bruzzese *et al.*, 1999). The amount of yttrium oxide in the ceramic has an essential role in the capacity of transformation of the tetragonal phase and, therefore, in the ceramic's toughness (Vleugels, Yuan *et al.*, 2002). This material consists of tetragonal grains, whose size is in the submicron scale, without a glassy phase at the edge of the crystals. The amount of tetragonal phase that remains at room temperature depends on many factors, such as grain size, yttrium oxide content, and the degree of constriction exerted upon them by the matrix (Covacci, Bruzzese *et al.*, 1999). Most commercial Y-TZP powders are manufactured using a process known as co-precipitation (Segal, 1997). The objective of this procedure is to manufacture multicomponent ceramic oxides through the formation of intermediate precipitates with the final goal of achieving an intimate mixture of the components during precipitation and keeping chemical homogeneity during calcination (Segal, 1997).

## 3. CONCLUSIONS AND FUTURE PERSPECTIVES

Today's ceramic materials used in the dental field comprise a large and diverse group of materials that offers patients a number of alternatives when dealing with prosthetic treatments.

These ceramic systems have been developed over time seeking highly-esthetic but also functional materials. Current dental ceramic systems (leucite or lithium disilicate-reinforced glass-ceramics, high-alumina or zirconia ceramics) offer better physical and mechanical properties than those of older, more traditional systems.

Mechanical values summarized in Table 5 show that zirconium oxide ceramics display the highest values of flexural strength under 3-point bending tests. When it comes to hardness, enamel and dentin display the lowest results when compared to all ceramic systems, and zirconium oxide ceramics exhibit the highest hardness value. The highest value for fracture toughness is showed by zirconium oxide ceramics, although dentin shows higher values than most ceramic systems and enamel displays lower values than dentin and ceramics.

Dental ceramics is an interesting area of research, since countless possibilities are opened for research, such as esthetics, processing of new ceramics with biological properties (increasing its interaction with cells or reducing bacterial adherence) and surfaces modifications processing.

## 4. ACKNOWLEDGMENTS

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## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

# Chapter 2. CERAMIC SUBSTRATES AND SILICA COATINGS PROCESSING

**Overview**

This chapter includes the ***third manuscript*** entitled *"The effect of slurry preparation methods on biaxial flexural strength of dental porcelain"*. It evaluates the effects of two mixing techniques on the biaxial flexural strength and microstructure of dental porcelain. This paper was published in The Journal of Prosthetic Dentistry.

In the present thesis, other aspects of the processing of dental ceramics and silica thin films modified with bioactive particles were studied. A selection of these results is showed in the Appendix A entitled "*Dental Ceramic Substrates*" and Appendix B entitled *"Silica Thin Films"*.

# Manuscript 3. THE EFFECT OF SLURRY PREPARATION METHODS ON BIAXIAL FLEXURAL STRENGTH OF DENTAL PORCELAIN



THE EFFECT OF SLURRY PREPARATION METHODS ON BIAXIAL FLEXURAL STRENGTH OF DENTAL PORCELAIN

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# THE EFFECT OF SLURRY PREPARATION METHODS ON BIAXIAL FLEXURAL STRENGTH OF DENTAL PORCELAIN

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## ABSTRACT

Statement of the Problem. One-step and incremental mixing procedures are currently used to produce dental ceramic pastes. In the ceramic industry, high quality is obtained using one-step mixing, but in dentistry, the best method has not been yet determined. Purpose. The purpose of this study is to evaluate the effects of 2 mixing techniques on the biaxial flexural strength and microstructure of dental porcelain. Material and Methods. Feldspathic porcelain discs (2×15 mm in diameter) were produced and divided according to the ceramic paste preparation method, powder-liquid incremental mixing group (n=50) or one-step mixing, as a control group (n=50). Specimens were tested for biaxial flexural strength and characterized using porosimetry, relative humidity, SEM/EDS, XRD, and FT-IR analyses. Statistical analysis was conducted using Weibull statistics. The Weibull modulus, characteristic strength and relative humidity were compared between groups, using Student's t-test and Mann-Whitney U test (α=.05). Results. The powder-liquid incremental mixing group showed significantly higher values (SD) of Weibull modulus (6.74 (0.700), *P*<.001) and characteristic strength (79.87

(2.01)) MPa, *P*<.001) when compared to the one-step mixing group (4.94 (0.94)) and (75.95 (2.6)) MPa). Significantly lower mean (SD) relative humidity values (*P*=.009) were found for powder-liquid incremental mixing group (20% (0.5%)) compared to one-step mixing group (22% (1%)). XRD spectra showed that the one-step mixing group produced higher amounts of the amorphous phase. Conclusions. Specimens produced by the incremental mixing technique showed higher biaxial flexural strength than one-step mixing. Clinical Implications. Ceramic slurry preparation may be a relevant factor in the performance of dental ceramics. A small variation in the relative humidity of green-state discs after de-molding, results in a decrease in Weibull modulus and characteristic strength for the one-step mixing method, which could be related to the presence of increased amounts of amorphous phase.

## 1. INTRODUCTION

Dental feldspathic porcelains are composed of an amorphous matrix ($K_2O$-$Al_2O_3$-$SiO_2$ glasses) with dispersed leucite particles and oxides, responsible for lowering the melting temperature of the material and providing color and opacity (JPD, 2005; ISO(6872), 2008). Low-fusing porcelains in fixed dental prostheses (FDP) exhibit desirable properties such as excellent esthetics (Sadowsky, 2006), high biocompatibility, (Messer, Lockwood *et al.*, 2003) high wear resistance, (Oh, Delong *et al.*, 2002; Schuh, Kinast *et al.*, 2005) and high chemical stability (Anusavice, 1992). However, their brittle behavior can jeopardize long-term durability of FDP, which can result in catastrophic failures under physiological or pathological loading during oral function (Kelly, Nishimura *et al.*, 1996; Rosenblum and Schulman, 1997; Jung, Wuttiphan *et al.*, 1999).

The long-term durability of porcelain restorations may be increased using several strategies that might be classified as preventive, to avoid the formation and propagation of large and heterogeneously distributed flaws (Oilo, 1988; Griggs, Thompson *et al.,* 1996; Griggs, Kishen *et al.,* 2003; Zhang, Griggs *et al.,* 2004; Cesar, Yoshimura *et al.,* 2005; Sinmazisik and Ovecoglu, 2006), thus, having direct effect on the mechanical properties; and corrective, focused on restoration repair procedures after failure (Galiatsatos, 2005; dos Santos, Fonseca *et al.,* 2006).

Dental porcelain processing involves several stages that must be carefully controlled to guarantee the final quality of the prosthetic work. After industrial production of high quality powders with controlled particle size and complex chemical composition, other important steps are performed by both the dental technician and the dentist, including the mixing of ceramic paste, followed by construction of the prostheses, sintering and polishing (Sinmazisik and Ovecoglu, 2006). Avoiding the introduction of defects during the manufacture of porcelain teeth is essential to obtain restorations with high mechanical properties, and therefore, longer clinical lifetimes (Fleming, Shaini *et al.,* 2000).

In the ceramic industry, optimized recipes are used to produce the porcelain slurry in which water is mixed with the porcelain powder in a highly controlled manner. In dentistry, porcelain slurries are also obtained by mixing water with the porcelain powder, however, an empirical approach is used in the laboratory to obtain a paste consistency that results in intra- and inter-operator variability (Palin, Fleming *et al.,* 2001; Zhang, Griggs *et al.,* 2004).

There are 2 different methods commonly used for preparing ceramic pastes in the dental laboratory, the incremental technique, in which water is added in small increments to the powder, and the one-step technique, in

which a predetermined amount of water is added in a single step to the porcelain powder. Both techniques aim at obtaining a final creamy porcelain slurry with an optimal powder/liquid ratio of 2.8 g/mL (Fleming, Shaini *et al.*, 2000; Zhang, Griggs *et al.*, 2004). When this optimal powder/liquid ratio is obtained, the liquid occupies small spaces between the ceramic particles and acts as a lubricant for particle movement, producing highly dense green-states and final fired-ceramics, (Kelly, Nishimura *et al.*, 1996; Zeng, Oden *et al.*, 1996; Cattell, Clarke *et al.*, 1997; Rosenblum and Schulman, 1997; Fleming, Shaini *et al.*, 2000; Palin, Fleming *et al.*, 2001; Zhang, Griggs *et al.*, 2004) with homogeneously distributed microdefects and uniform porosity (Gill, 1932; Anusavice and Hojjatie, 1987; Fleming, Shelton *et al.*, 1999).

Excess water in the porcelain slurry is a problem, because excess water in the cast will evaporate and result in higher porosity. Moreover, excess water may react with some components of the porcelain and induce changes in the microstructure of the final fired product, changing the relative amounts of crystalline and glassy phases (Tomozawa, Han *et al.*, 1991; Ray and Day, 1996; Mackert Jr, Williams *et al.*, 2000; Burgner and Weinberg, 2001; Deubener, Muller *et al.*, 2003).

Weibull statistics is a commonly used method for characterizing the failure of brittle materials under different flexural configurations (Oilo, 1988; Seghi, Daher *et al.*, 1990; Quinn and Quinn, 2010). The Weibull distribution is an extremely valuable distribution with descriptors such as Weibull modulus (a dimensionless dispersion measurement) and characteristic strength (stress value at which the probability of failure (Pf) is 63.2%). The Weibull distribution is homologous to the descriptors used in normal distribution such as standard deviation (a dispersion measurement) and mean (a stress value where the Pf is 50%) values (Quinn and Quinn, 2010). The accuracy of

the Weibull analysis for dental ceramics depends on the quality of the specimens tested, as well as proper selection of the test design and algorithms to estimate the descriptors based on the obtained data (Quinn and Quinn, 2010). A high Weibull modulus is advantageous, because it indicates a low structural variability among specimens and a more predictable failure behavior (Ritter, 1995a). A low structural variability of the fired ceramic restorations, at least theoretically, might reduce the incidence of fractures and chips, therefore reducing the need for repair and replacement of FPR.

The purpose of the study was to evaluate the biaxial flexural strength and microstructure of porcelain specimens obtained by 2 different slurry preparation methods (powder-liquid incremental and one-step mixtures). The hypothesis was that there would be significant differences between the Weibull parameters calculated from biaxial flexural strength of specimens obtained by the different preparation techniques.

## 2. MATERIAL AND METHODS

In this study low fusing porcelain (Vita Omega 900, Dentin, Color 4R 1.5; Vita Zahnfabrik, Bad Sackingen, Germany) was used to produce discs 15 mm in diameter and 2 mm thick. The specimens were divided in 2 groups, according to the preparation method. Powder-liquid incremental group discs (n=50) were obtained by incremental addition of the liquid to the powder using a dental laboratory brush (#8; Smile Line, St-Imier, Switzerland). One-step group discs (n=50) were obtained by single addition of the liquid to the powder using 1.82 g of powder per 0.66 mL of distilled water (Zhang, Griggs *et al.*, 2004). All discs were fabricated using a 18 mm diameter plastic syringe (Becton Dickinson & Co, Franklin Lakes, NJ), which was sectioned and its plunger was polished and used as the disc base (Giordano, Pelletier *et al.*, 1995).

In both methods, the ceramic paste was prepared at room temperature on a glass tile and then loaded into the syringe with a ceramic brush; the ceramic was condensed using an absorbent paper towel to remove the excess water (Giordano, Pelletier *et al.*, 1995). The plunger of the syringe was placed 3 mm away from the top-edge using a periodontal probe. The surface of the disc was leveled against a flat surface to ensure a uniform thickness before removing the disc from the syringe (Zhang, Griggs *et al.*, 2004). The green-state specimens were removed from the syringe with a scalpel and placed on a refractory platform (Vita Zahnfabrik). All discs were heat-treated in an oven (Vacumat 40; Vita Zahnfabrik) according to the procedure described by the manufacturer. After sintering, the surfaces of the discs were polished with a series of abrasive papers (400, 600, and 1200 grit) using a polishing machine (LaboPol-5; Struers, Copenhagen, Denmark) (Zeng, Oden *et al.*, 1996).

Biaxial flexure strength was determined with a ball-on-ring design, (Fleming, Shelton *et al.*, 1999; Fleming, Shaini *et al.*, 2000) where each disc was supported on a steel ring (9.86 mm diameter). Load was applied centrally through a spherical ball indenter (5 mm diameter) in a universal test machine (650R; TestResources Inc, Shakopee, Minn) with a crosshead speed of 0.5 mm/min until failure. The maximum strength of the biaxial flexure test for each disc was calculated according to (Fleming, Shelton *et al.*, 1999):

$$\sigma_{max} = ( P/h^2 ) \{ (1 + \nu) [ 0.485 \times \ln(a/h) + 0.52] + 0.48\}$$

where $\sigma_{max}$ is the maximum tensile strength (MPa), $P$ is the measured load at fracture (N), $h$ is the thickness of the disc (mm), $\nu$ is the Poisson's ratio (0.25 for porcelain), (Fleming, Shaini *et al.*, 2000) and $a$ is the radius of the supporting ring (mm).

The Weibull distribution was used to calculate fracture probabilities of each group as a function of applied stress. The equation used for the Weibull - parameter distribution function was: (Kelly, 1995)

$$P_f = 1 - \exp\{-\int_S [(\sigma/\sigma_0)]^m dS\}$$

Where σ is stress; $\sigma_0$ is scale parameter or characteristic strength (stress level at which 63.2 % of the specimens failed); *S* is the surface under stress; and *m* is a dispersion parameter or Weibull modulus related to the slope of the distribution.

Specimens obtained by the different processing methods were characterized in terms of chemical composition, powder/liquid ratio, fractography, crystallinity and porosity. These analyses were performed for the commercial raw powder, green-state and sintered discs. To quantify the relative humidity after de-molding, 5 green-state discs per group were tested at 180° C using a thermal infrared moisture balance (Eurotherm, Gibertini Elettronica; Milano, Italy), which has an automatic function to determine the stable weight (DSW). The DSW value is defined when the variation in the weight is lower than 0.01 mg for 9 minutes.

Compositional analysis of the raw powders was accomplished using x-ray fluorescence (PW2404 X-ray Spectrometer; PANalytical B.V., Almelo, The Netherlands) and atomic absorption spectroscopy (Solar 929 AA Spectrometer; ATI Unicam, Cambridge, UK). For Fourier transformed infrared (FT-IR) analysis, fractured discs were evaluated using a spectrometer (System 2000; PerkinElmer, Wellesley, Mass) (Griggs, Kishen *et al.,* 2003; Griggs, Wataha *et al.,* 2003; Bertolini, Zaghete *et al.,* 2004) with an attenuated total reflectance accessory (Golden Gate Single Reflection Diamond ATR; Specac, Smyrna, Ga). For the FT-IR characteristic spectrum of both groups, 3 discs fractured under biaxial flexural test were selected from the tenth percentile where the fracture was obtained at lowest strain. In each disc, at

least 3 spectra were obtained at different areas (each spectrum is the average of 100 scans between 400 $cm^{-1}$ to 4000 $cm^{-1}$ with 4$cm^{-1}$ resolution). Baseline correction and mean spectrum were then calculated using numerical software (Matlab, Version R2008b; Mathworks Inc, Natick, Mass).

Two different types of surfaces, fractured and polished, (both etched with 2% HF hydrofluoric acid) (Cesar, Yoshimura *et al.,* 2005) of 3 discs (within the tenth lowest percentile strength) of both groups were examined using scanning electron microscopy (SEM) and energy dispersive spectroscopy (Gotfredsen, Hjorting-Hansen *et al.*) microprobe (JEOL JSM-6301F, Tokyo, Japan). X-ray diffraction (XRD) was used to identify the crystalline phases and detect the presence of amorphous phases in specimens using the 2Θ-angle range between 5 and 85 degrees with a scan speed of 0.02 degrees/s (X´Pert PRO alpha-1, PANalytical B.V., Almelo, The Netherlands), using a diffracted x-ray detector (RTMS X´Celerator; PANalytical B.V.) The XRD characteristic spectrum was obtained from the mean of 3 discs per group in the tenth lowest percentile, using dedicated software (High Score Plus XRD; Version 2.2, PANalytical B.V.). The detected peaks were matched using the crystallographic database of International Centre for Diffraction Data (ICDD) in the Powder Diffraction File (PDF, 2004) (Tsetsekou, Papadopoulos *et al.,* 2002; Sinmazisik and Ovecoglu, 2006). Volume open porosity was calculated following the determination of the apparent density based on the Archimedes water displacement method (Gonzaga, Cesar *et al.,* 2008).

Statistical analysis was conducted using a descriptive approach based in the maximum and minimum values of calculated biaxial flexural strength. The Kolmogorov-Smirnov (KS) test was applied to determine if the Weibull model was an adequate distribution for the experimental data obtained for both groups. The estimation of the Weibull parameters (m, $\sigma_0$) was calculated

by the maximum likelihood method using statistical software (R, v2.11.1, R Foundation for Statistical Computing, Vienna, Austria) (Ritter, 1995b; Pelaez, Garcia *et al.*, 2004; Pham, 2006). Weibull modulus, characteristic strength, relative humidity and porosity values were compared using 2-tailed Student's t-test or Mann-Whitney test, depending on the evaluated normality of distribution according to KS test. All comparisons were conducted at $\alpha = .05$.

## 3. RESULTS

Descriptive statistical analysis showed that mean (SD) biaxial strength values were 74.61 (12.71) MPa for the powder-liquid incremental mixture group and 69.72 (16.35) MPa for the one-step group. To test that the experimental data fit a Weibull distribution, the data were compared with a theoretical distribution and both groups showed no statistically significant deviations from the theoretical distributions $P=0.528$ for one step process and $P=0.685$ for incremental process), confirming that the data could be modeled as a Weibull distribution.

The Weibull moduli (SD) obtained in the present work were relatively low but the values for the powder-liquid incremental mixing group (6.74 (0.70)) were significantly higher ($P<.001$) than those obtained for the one-step mixing group (4.94 (0.94)). In addition, the characteristic strength (SD) values found were also significantly higher ($P<.001$) for the powder-liquid incremental mixing group (79.87 (2.01)) MPa) than for the one-step mixing group (75.95 (2.61)) MPa).

The results of chemical analysis by x-ray fluorescence confirmed that the raw porcelain powder has the typical composition of conventional dental porcelains, that is 60% $SiO_2$; 15% $Al_2O_3$; 8% $K_2O$ and 6% $Na_2O$, with smaller amounts of other oxides such as CaO, $TiO_2$, MgO, $SnO_2$, BaO and $LiO_2$ that

were confirmed by atomic absorption spectroscopy. Significantly higher ($P$=.009) mean (SD) values of the relative humidity in green-state discs after de-molding were found for the one-step group (22% (1%)) compared to the powder-liquid incremental mixing group (20% (0.5%)).

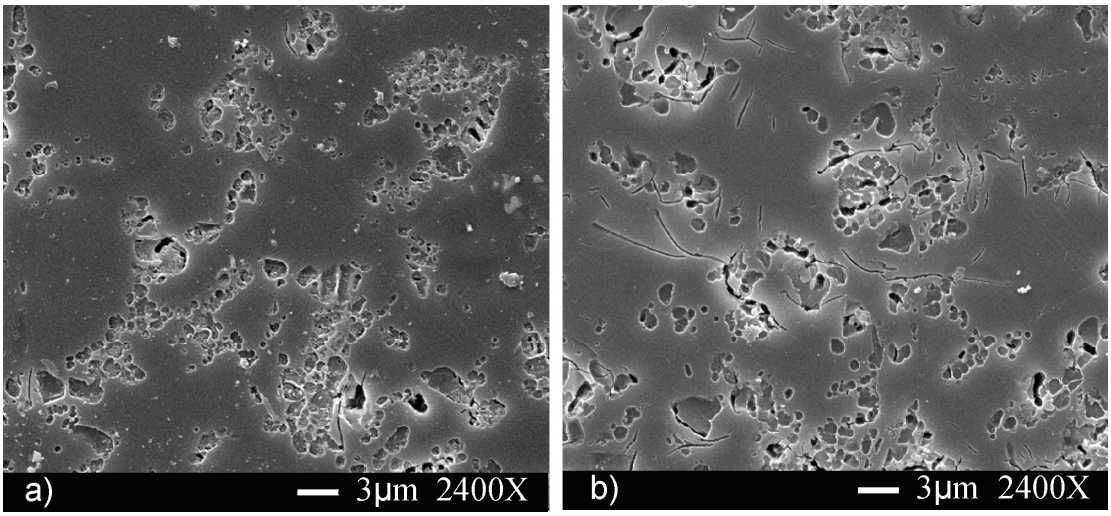



Figure 9. Characteristic SEM images of specimens polished and HF 2% treated. (a) Powder-liquid incremental and (b) one-step mixing groups (×2400).

No obvious morphological differences were observed in the SEM analysis between fractured discs derived from both preparation methods. In the evaluation of the polished sections (Figure 9), a small qualitative difference was noticed in the porosity. However, no significant differences ($P$=.916) were found in the volume open porosity test (full data not shown) between both experimental groups.

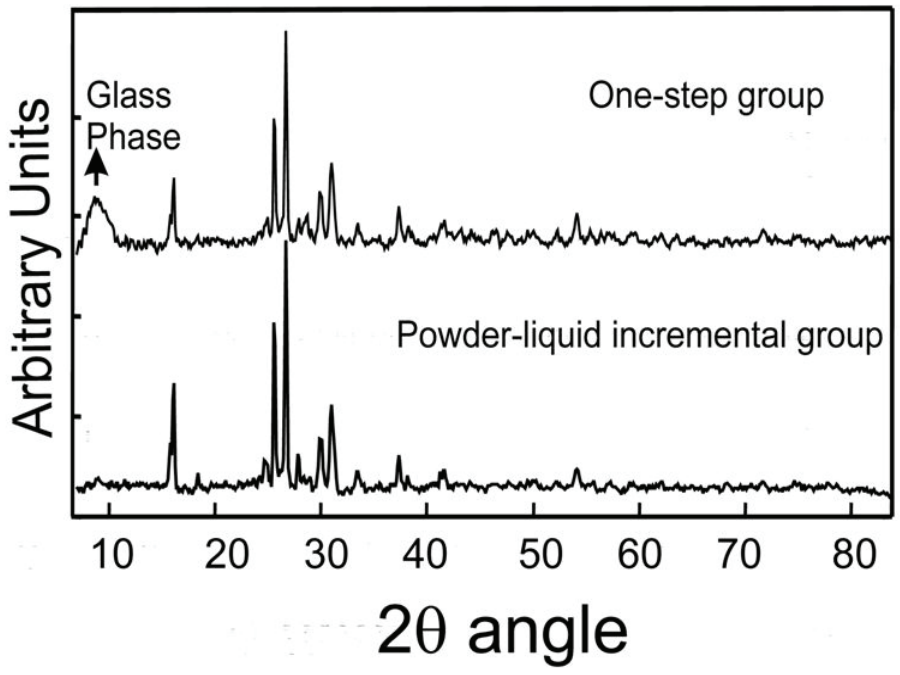



Figure 10. X-ray diffraction spectra of sintered dental porcelain discs produced by 2 different mixing methods:
a) one-step group and b) powder-liquid incremental group.

Figure 10 shows that XRD characteristic spectrum for both groups exhibited the same diffraction pattern of the crystalline phase (identified as cubic Leucite). In addition, a larger amount of amorphous phase was detected in discs produced by one-step mixing. Figure 11 shows a comparison of the FTIR attenuated reflectance spectra for powder-liquid incremental and one-step mixing groups. IR absorption bands with peaks at 430, 710, 970, 1640, 2920 $cm^{-1}$; as well as a broad absorption band from 3150 to 3650 $cm^{-1}$, were observed for both groups.

## 4. DISCUSSION

The research hypothesis that significant differences would be found between the Weibull parameters calculated from biaxial flexural strength of discs produced by 2 different slurry preparation methods was accepted, since

the powder-liquid incremental method resulted in significantly higher Weibull modulus and characteristic strength compared to the one-step technique. The higher Weibull modulus obtained by the powder-liquid incremental method indicates a lower variability of strength values in this group and consequently higher reliability of the structures produced by this technique. The higher characteristic strength obtained for the incremental group indicates that higher stress levels are necessary to cause the fracture of 63.2% of the specimens produced using this technique.

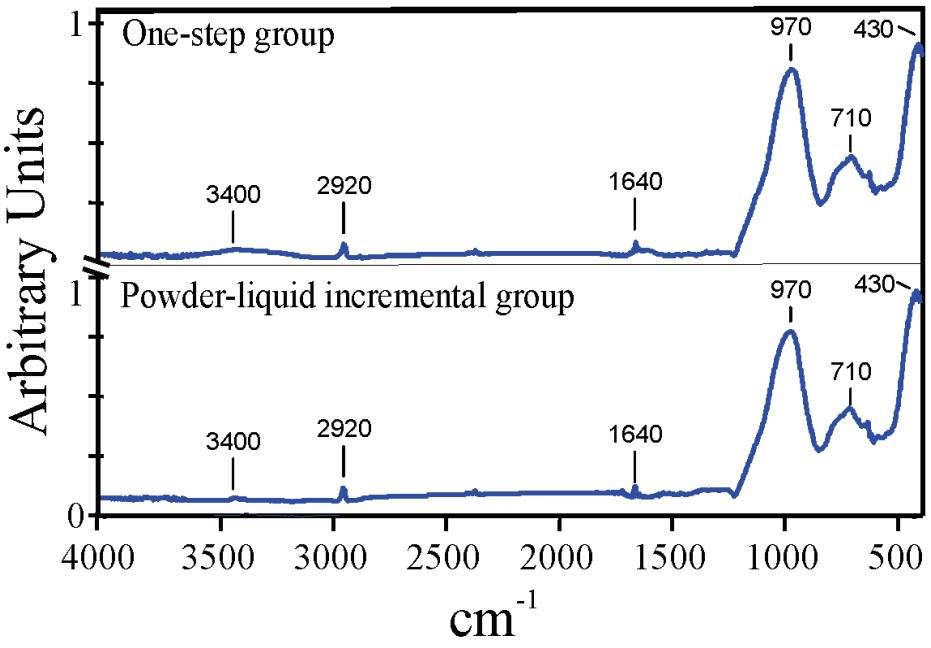



Figure 11. Fourier transformed infrared attenuated reflectance spectra (absorbance mode)

Sintered dental porcelain discs produced by 2 different mixing methods.

The better mechanical behavior of the porcelain specimens produced by the powder-liquid incremental technique is most likely due to the fact that this method results in a lower amount of pores in the porcelain structure compared to the one-step method. The relative humidity analysis showed that higher amounts of liquid were found in the green-state discs of the one-step

group. Therefore, it is clear that when water is added in a single step to the porcelain powder, larger amounts of liquid are retained in the spaces between the powder particles. As a consequence, higher porosity is expected due to the larger amount of space between powder particles following the evaporation of the water, reducing the efficacy of the viscous flow sintering process and leading to higher porosity levels (Gill, 1932; Fleming, Shelton *et al.*, 1999).

The porosity level may not be the sole factor affecting the flexural strength of dental ceramics, since a previous study (Gonzaga, Cesar *et al.*, 2008) showed that glass-ceramics with significantly higher porosity (due to processing at higher temperatures) possessed statistically similar flexural strengths to glass-ceramics with lower porosities. It is important to consider that the flexural strength of brittle materials is determined by the flaw population of the tested material, and therefore, the present study showed that the one-step technique produced in a less favorable flaw population resulted in decreased mechanical behavior.

Another possible explanation for better mechanical behavior of specimens produced by the incremental group is the lower amount of amorphous phase in these specimens, as shown in the XRD analysis. The amorphous phase has been shown to be the weakest part of dental porcelains, since they are more susceptible to water-assisted degradation (Griggs, Wataha *et al.*, 2003). Also, a low amount of amorphous phase indicates that other phases are more abundant, such as the leucite phase, which can increase the fracture toughness of dental porcelains by the crack deflection toughening mechanism (Cesar, Yoshimura *et al.*, 2005). It is not obvious how the water content of the green-state specimen affects the phase distribution in the final material. The sintering of dental porcelain within the furnace is a complex viscous flow process and the crystallization of leucite within the glassy matrix

is governed by many factors such as temperature, heating rate, and powder characteristics (Tsetsekou, Papadopoulos *et al.,* 2002).

After sintering, FT-IR spectroscopy was used to evaluate the presence of hydroxyl and molecular water in the bulk discs, since their presence has been associated with a higher relaxation of the glass structure (Tomozawa, Han *et al.,* 1991). A broad band with peak at 3400 $cm^{-1}$ appeared higher in the one-step group compared to the incremental group, which might be interpreted as a higher level of hydroxyl water. However, further studies are necessary to confirm this trend and to understand how excess water may affect the crystalline content of these materials. Other IR absorption peaks and bands showed comparable results for discs from both mixture techniques evaluated.

They were determined to correspond to H-O-H bonds (1640 $cm^{-1}$), (Griggs, Kishen *et al.,* 2003; Griggs, Wataha *et al.,* 2003) Si-O-Si bonds (710$cm^{-1}$, the bringing vibration of oxygen) (Griggs, Kishen *et al.,* 2003; Griggs, Wataha *et al.,* 2003) within the glass network, O-Al-O bonds (430 $cm^{-1}$ corresponds to vibration of oxygen), (Bertolini, Zaghete *et al.,* 2004) Si-O- associated with alkaline ions, such as $Na^{+}$, $K^{+}$ and $Ca^{2+}$ in the glass network (a broad peak from 760 to 970 $cm^{-1}$ results from the combined vibrations of non-bridging oxygen), (Bertolini, Zaghete *et al.,* 2004) and O-C-O bonds (2920 $cm^{-1}$ corresponds to vibration of oxygen), a group normally observed in this technique.

Authors of another study (Fleming, Shaini *et al.,* 2000) that used a different methodology to vary the amount of water added to the porcelain powder presented results that agree with this study, since it was demonstrated that feldspathic porcelain (Vitadur-Alpha) had its Weibull

modulus reduced when a higher amount of water than an optimal powder-liquid ratio of 2.8 was added to the porcelain powders. The same authors (Fleming, Shelton *et al.,* 1999) studied aluminous porcelain (Vitadur-N) and found that deviating from an optimal powder-liquid ratio (either higher or lower) resulted in a decreased Weibull modulus. These authors indicated that it is important to produce porcelains with relatively low apparent porosity as it would also be expected to reduce the amount of shrinkage on firing and as a consequence improve the marginal fit of porcelain in practice.

In the present study, the powder/liquid ratio for specimen preparation was selected according to other reports so that one-step mixing group results could be compared with those from other studies (Fleming, Shaini *et al.,* 2000; Zhang, Griggs *et al.,* 2004). Absorbent paper towels were used to remove excess water in the ceramic paste for both groups (Giordano, Pelletier *et al.,* 1995). Removal of the liquid with an absorbing tissue allows the particles to move closer together under the action of the surface tension of the liquid (Fleming, Shaini *et al.,* 2000; Zhang, Griggs *et al.,* 2004). Such powder condensation aims at achieving the maximum packing density for the powder, and light vibration techniques are used to reorient the powder particles into a position of maximum packing efficiency by eliminating as much excess liquid as possible (Fleming, Shelton *et al.,* 1999; Fleming, Shaini *et al.,* 2000). However, this procedure also added a further variability in the preparation of green-state discs. Another aspect related with specimen preparation in the present study is that the discs were polished and not glazed to better control the final disc thickness and to reduce the complexity of the studied model; however, this procedure probably exposed pores and increased surface roughness (Quinn and Quinn, 2010).

The results of the present study are clinically relevant because they showed that dental technicians should be encouraged to use the powder-

liquid incremental technique presented for the production of ceramic prostheses with higher structural homogeneity and better mechanical properties. However, some limitations of the present work should be considered, such as the use of only one commercial porcelain powder, as different results may be obtained with different porcelains or ceramic compositions. Also, the number of specimens could have been increased to improve the statistical analysis of the characterization by high resolution techniques. Finally, direct extrapolation to the clinical situation should be carefully considered as this study did not investigate other factors present in the oral cavity such as pH variations, thermal cycling or occlusal forces.

## 5. CONCLUSIONS

Considering the limitations of this *in vitro* study, the results showed that the incremental technique for the preparation of porcelain slurry resulted in lower water content in the green-state specimens, and compared to the one-step technique, produced significant improvements in both the Weibull modulus and characteristic strength of the porcelain tested. Microstructural analyses showed that observed improvements in the mechanical properties of the specimens prepared by the incremental technique may be related to decreased porosity and increased crystalline content.

## 6. ACKNOWLEDGMENTS

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OH), Professor Derek Hansford (The Ohio State University, Columbus, OH) for helpful discussion.

## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

# Chapter 3. BIOLOGICAL BEHAVIOR ON MICROFABRICATED SILICA COATINGS

**Overview**

This chapter is divided in six manuscripts:

***Fourth manuscript***, entitled *"Cells spreading on micro-fabricated silica thin film coatings"*, describes the production of pillar-shape micropatterned silica coatings on glass substrates, and evaluates the adhesion of HBMSCs during an initial period (4h-24h). This extended abstract was published in a Special Issue of Microscopy and Microanalysis.

***Fifth Manuscript***, entitled "*Early spreading and propagation of human bone marrow stem cells on isotropic and anisotropic topographies of silica thin films produced via microstamping"*, describes the production of isotropic and anisotropic microfabricated silica coatings obtained by sol-gel and to compares the biological behavior of HBMSCs on these surfaces at early stages of adhesion and propagation. This full paper was published in Microscopy and Microanalysis.

***Sixth Manuscript***, entitled *"Cellular alignment induction during early in vitro culture stages using micropatterned glass coatings produced by sol-gel process"*, compares the cell adhesion and early orientation of HBMSCs cultured on micropatterned and on flat glass coatings produced by sol-gel. This paper was published in Key Engineering Materials.

***Seventh Manuscript***. *"Microfabricated $SiO_2$ thin films on structural dental ceramics"* describe the development of a technique to obtain microstructured

$SiO_2$ coatings on 3Y-TZP based on soft lithography micropatterning and sol-gel processing. This paper was published in Bioceramics 22 – Proceedings of 22nd International Symposium on Ceramics in Medicine. In the sequence of this work the following paper was published.

***Eighth Manuscript***. *"Isotropic micropatterned silica coatings on zirconia induce guided cell growth for dental implants",* this work the following paper was published to promote guided tissue regeneration on zirconia materials for application in dental implantology. This full paper was published in Dental Materials.

Other aspects of the biological behavior on coatings modified with bioactive particles (hydroxyapatite and Portland cement) were studied. A selection of these results is showed in the Appendix B entitled entitled *"Silica Thin Films"* and Appendix C *"In vitro Cell Behavior"*.

# Manuscript 4. CELLS SPREADING ON MICRO-FABRICATED SILICA THIN FILM COATINGS

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doi:10.1017/S1431927

## Cells spreading on Micro-fabricated Silica Thin film Coatings

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78. doi:10.1017/S1431927609990845

# CELLS SPREADING ON MICRO-FABRICATED SILICA THIN FILM COATINGS

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## ABSTRACT

From a biomaterials perspective, it is now understood that success in the osseointegration of a dental implant is conditioned by its "macro", "micro" and "nano" scale features. Macro-scale roughness is necessary to improve primary stabilization in the post-surgical phase inducing a peri-implant thin fibrous layer. However, the more complex process in the true cell-material interaction is dependent on micro and nano scale phenomena (Boyan , Lohmann *et al.*, 2001). There is clear evidence that cell adhesion, proliferation, organization and phenotype are modulated at the micro-scale and that protein absorption is fundamentally a process conditioned at nano-scale (Wennerberg, Albrektsson *et al.*, 1995).

Hypothetically, a biologically friendlier surface may be produced using soft lithography and sol-gel techniques to obtain micro-structured coatings for dental applications. The purpose of this work was to compare spreading of Human Bone Marrow (HBM) cells on silica thin films produced by sol-gel, processed with two different surface features: micro-pillars patterns (MPPS) and flat surfaces (FS) (Malmstrom, Slotte *et al.*).

Sol-gel processing was used to produce hybrid silica sols resulting from acid catalysis in a single stage. Tetraethylorthosilicate (TEOS, Aldrich) and Methyltriethoxisilane (MTES, Aldrich) were selected as silica precursors for the sol, while alcohol was used as a solvent and nitric acid and acetic acid were used as catalysts. Spin Coating technique was chosen to apply $SiO_2$ flat coatings on microscope glass slides as model substrates.

Photolithography was used to produce master pattern with micro-scale dimensions. A polydimethylsiloxane (PDMS) molding was obtained from the masters by uniformly mixing PDMS (Silastic T-2, Dow Corning, USA) with a curing agent, pouring the mixture onto the masters, degassing, and curing (Gallego, Ferrell *et al.,* 2008). A molding was used to stamp $SiO_2$ coatings produced by a sol-gel process. The coatings were sintered using a thermal cycle that included heating up to 450ºC at a rate of 20ºC/min, a plateau at 450ºC for 30 min and cooling in air using an elevator furnace, followed by autoclave sterilization.

Human Bone Marrow (HBM) was obtained from patients undergoing orthopaedic surgery procedures. Primary cultures of Human Bone marrow was cultured under a standardized protocol and maintained for 10/15 days till near confluence, when adherent cells were enzymatically released. Flat and micro-patterned coatings were cultured with HBM cells ($3^{th}$ and $5^{th}$ passage), under the same conditions of Primary cultures for 4h and 24h. Samples were prepared according to the following sequence: Three cultured samples per group were washed with PBS, fixed using 1.5% glutaraldehide, dehydrated in graded alcohol solutions, gold sputtered. Cell morphology and orientation were evaluated by SEM.

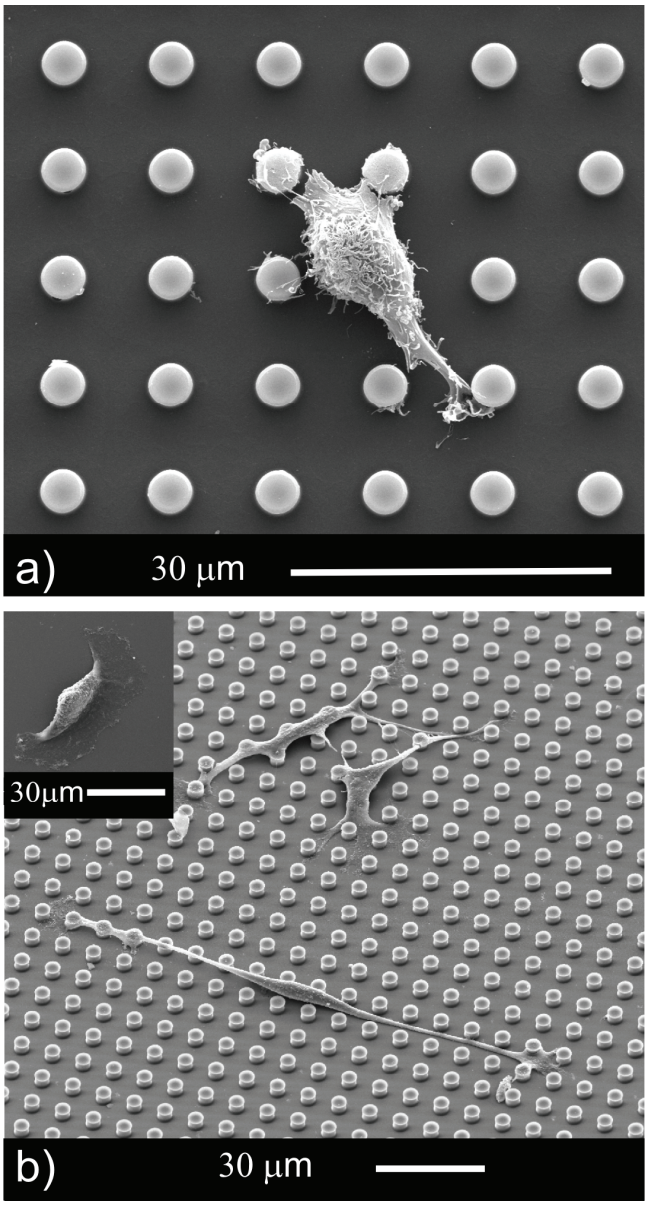



Figure 12. Fig. 1. SEM images. (SE mode, 15kV) of HBM cells on MPPS Silica coatings after 4h incubation.

a) Typical cell showing a rounded shape with filipodium and incipient cytoplasmatic extensions attached in the top and base of pillars. b) Images of HBM cells on Silica coatings after 24h incubation. General view showed include a multipillar structured silica surface where adherent cells adopted a slimmer shape with long cytoplasmatic extensions. In the Upper-Left close view HBM cell with a butterfly shape and multidirectional spreading on Flat Silica surface produced by Spin Coating.

FS (thickness ~1.5 μm) and MPPS (5 μm periodicity) coatings were obtained. After 4h incubation, HBM cells exhibited well-defined morphology with an incipient spreading on the structured surface of MPPS silica coating (Figure 12a). After 24h incubation, cells had two typical morphologic features: (a) Elliptic slimmer shape, with a long cytoplasmatic extensions growing on the top of pillars or inside the valleys between adjacent pillars (Figure 12b) butterfly shape (Figure 12b -Inset) with multidirectional spreading on FS silica coatings. MPPS surfaces didn't show a clear control over HBM cell

orientation. Previous studies on HBM cells behavior on line shaped micro-patterned coatings had shown contradictory results, with a marked controlled cell orientation being present (Pelaez-Vargas, Ferrell *et al.*, 2009b).

## ACKNOLEDGMENTS

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## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

# Manuscript 5. EARLY SPREADING AND PROPAGATION OF HUMAN BONE MARROW STEM CELLS ON ISOTROPIC AND ANISOTROPIC TOPOGRAPHIES OF SILICA THIN FILMS PRODUCED VIA MICROSTAMPING

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## Early Spreading and Propagation of Human Bone Marrow Stem Cells on Isotropic and Anisotropic Topographies of Silica Thin Films Produced via Microstamping

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# EARLY SPREADING AND PROPAGATION OF HUMAN BONE MARROW STEM CELLS ON ISOTROPIC AND ANISOTROPIC TOPOGRAPHIES OF SILICA THIN FILMS PRODUCED VIA MICROSTAMPING

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## ABSTRACT

While there has been rapid development of microfabrication techniques to produce high resolution surface modifications on a variety of materials in the last decade, there is still a strong need to produce novel alternatives to induce guided tissue regeneration on dental implants. High resolution microscopy provides qualitative and quantitative techniques to study cellular guidance in the first stages of cell material interactions. The purposes of this work were 1) to produce and characterize the surface topography of isotropic and anisotropic microfabricated silica thin films obtained by sol-gel processing, and 2) to compare the *in vitro* biological behavior of Human Bone Marrow Stem cells (HBM) on these surfaces at early stages of adhesion and propagation. The results confirmed that a microstamping technique can be used to produce isotropic and anisotropic micropatterned silica coatings.

AFM analysis was an adequate methodology to study in the same specimen the sintering derived contraction of the microfabricated coatings, using images obtained before and after thermal cycle. Hard micropatterned coatings induced a modulation in the early and late adhesion stages of cell material and cell cell interactions in a geometry dependent manner (i.e. isotropic vs. anisotropic), as it was clearly determined, using scanning electron and fluorescence microscopies.

## 1. INTRODUCTION

Dental Implants as an alternative treatment for lost teeth are not free of controversy (Sennerby, 2008), and important differences may be found when comparing experimental and clinical results (Albrektsson, Sennerby *et al.*, 2008).

In general, it is well accepted that macro-roughness provides primary stabilization for dental implants in the post-surgical phase, and that slight micro-roughness has some clinical advantages (Wennerberg, Albrektsson *et al.*, 1995; Albrektsson and Wennerberg, 2004). However, results of these studies are often not statistically significant. Recently, the interest in nanoscale roughness is growing (Meirelles, Currie *et al.*, 2008), as protein adsorption occurs at this scale, which is a key step in the process of osseointegration. To date, no randomized controlled clinical trial has been reported supporting superior behavior for features at this scale.

Small-scale features on the dental implant surfaces are produced using additive or subtractive techniques, leading to random topography and heterogeneous chemistry. The main disadvantages presented by additive approaches are low coating adhesion, cohesive failures due to increased

thickness and high temperature/vacuum requirements (Silva, Santos *et al.*, 1998).

Sol-gel chemistry and soft lithography, as synergistic techniques, could be interesting alternatives to other surface modification techniques (Lee, Lee *et al.*, 2005; Nayab, Jones *et al.*, 2005; Perrie, Rushton *et al.*, 2005; Zinger, Zhao *et al.*, 2005; Teixeira, Monteiro *et al.*, 2007; Vorobyev and Guo, 2007). It is well understood that flat silica coatings with homogeneous chemistry can be produced, using a low cost sol-gel process and low sintering temperatures (Gallardo, Galliano *et al.*, 2000a; Duran, Conde *et al.*, 2004; Pelaez, Garcia *et al.*, 2004). Soft lithography is an inexpensive technique, amenable to a wide range of materials and processing environments, that can be used to create micro- and nano-discontinuities on a surface in a controlled, high throughput manner (Xia and Whitesides, 1998). The combination of soft lithography and sol-gel processing (Marzolin, Smith *et al.*, 1998) has not been extensively studied for the modification of ceramic materials, but it presents an interesting alternative for ceramics implants to induce guided tissue regeneration.

Cells are sensitive to their surrounding environment, and the effects of random or well-organized micro- or nano-topography are the subjects of continued research, allowing for many combinations of chemistry, surface topography, types of cells, and clinical applications (Rhee, Taylor *et al.*, 2005; Gallego-Perez, Higuita-Castro *et al.*, 2010). The effects of topography on cells can be evaluated, in terms of adhesion, as variations in the morphology, alignment, elongation, cytoskeleton and focal contact formation; and the effects on metabolism, in terms of transcriptional activity, gene expression and the production of extracelular matrix (Healy, Thomas *et al.*, 1996; Mata, Boehm *et al.*, 2002; Tan and Saltzman, 2004; Wang and Ho, 2004; Baudino, McFadden *et al.*, 2008; Pelaez-Vargas, Ferrell *et al.*, 2009a; Kim, Boehm *et al.*, 2010).

When cells-material interactions are tested on a new material, a fundamental approach is to assess cell adhesion, spreading and propagation on the surface through an imaging-based process. High resolution techniques allow for the observation of structures (dorsal filipodia, laminopodia) that are ignored by other techniques (Bohil, Robertson *et al.,* 2006), and cell to cell analysis is possible. Complementary biochemistry and molecular biology techniques are also required. This approach enables a combination of qualitative and quantitative analysis of the response (Curtis, 2001; Dalby, Riehle *et al.,* 2004; Wang and Ho, 2004; Mata, Kim *et al.,* 2009; Kim, Boehm *et al.,* 2010).

The purposes of this work were 1) to produce and characterize the surface topography of isotropic and anisotropic microfabricated silica thin films obtained by sol-gel processing, and 2) to compare the *in vitro* biological behavior of Human Bone Marrow Stem cells (HBMSCs) on these surfaces at early stages of adhesion and propagation.

## 2. MATERIALS AND METHODS

A hybrid silica sol was produced under acidic conditions using sol-gel technology. Two silica precursors were selected, tetraethylorthosilicate (TEOS, Aldrich, Milwaukee, WI, USA) and methyltriethoxisilane (MTES, Aldrich, USA) in a 40:60 molar ratio. Ethanol was used as a solvent and nitric and acetic acids were used as catalysts. Synthesis was done under reflux for 3h. An early aging was completed after 24 h at 4 °C. A spin coater was used to apply a thin film on glass cover slips for 45s at 3000 rpm. Non-coated glass slides were used as a control group.

A two-stage microstamping process was implemented to fabricate silica micropatterns. Initially a standard photolithography process was used

to pattern a photoresist on a silicon wafer, and produce masters with multiple geometries and micro-scale dimensions. The soft lithography process included the production of a polydimethylsiloxane (PDMS, Silastic T-2, Dow Corning, Midland, MI, USA) mold from the silicon master, following a process that included mixing, casting, degassing and curing (Gallego, Ferrell *et al.*, 2008). Silica was drop-cast on the coverslips and brought into contact with the PDMS mold under pressure (2.7 kPa) for 2h, then de-molded and sintered at 500 °C for 30 min. This process was used to obtain arrays of 5 μm wide lines (anisotropic pattern) and 5 μm diameter pillars (isotropic pattern), with 5 μm spacing edge-to-edge (Figure 13).

The effects of sintering on the dimensional stability of microfeatures in the coatings were evaluated using atomic force microscopy (Veeco Metrology Multimode/Nanoscope IVA, Plainview, NJ, USA) in tapping mode. AFM images were acquired before and after sintering treatment. Roughness was analyzed in terms of amplitude and spatial parameters using SPIP software (Image Metrology, Denmark). Six boxes of 3x20 μm in each image were analyzed, on the top surfaces and on bottom surfaces of micropatterned silica coatings.

The surfaces of the PDMS mold and silica micropatterned coating were analyzed using a field emission scanning electron microscopy (FEI Quanta 400FEG ESEM, The Netherlands) and EDS (energy dispersive spectroscopy) microprobe (EDAX Genesis X4M, Mahway, NJ, USA).

Bone marrow was obtained from patients undergoing orthopedic surgery procedures. Informed consent to use this biological material, that would be otherwise discarded, was obtained. Bone marrow was cultured in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum, penicillin-streptomycin (100 IU/ml and 10 mg/ml, respectively), fungizone

(2.5 µg/ml) and ascorbic acid (50 µg/ml). Cultures were incubated in a humidified atmosphere of 5% $CO_2$ in air at 37 °C, and the medium was changed twice a week. Primary cultures were maintained for 10/15 days until near confluence, and the adherent cells were enzymatically released with 0.04% trypsin and 0.025% collagenase. The resultant cell suspension was cultured ($2x10^4$ cell/$cm^2$) under the same experimental conditions on the isotropic and anisotropic silica micropatterns, but the culture medium was further supplemented with 10 mM β-glycerolphosphate and 10 nM dexamethasone. Cell cultures were routinely monitored by phase contrast optical microscopy and characterized by SEM, at different time points (4h, 24h, 7 day and 14 day). For SEM analysis, cells were washed twice with warm PBS, fixed in 10% v/v neutral buffered Formalin for 15min, dehydrated in graded ethanol solutions (50% to 100%) and Critical Point dried. Samples were gold sputtered prior to imaging.

For morphology evaluation via fluorescence inverted microscopy (Eclipse TE2000-5, Nikon, Japan), the cells were washed and permeabilized with 0.1% v/v Triton X-100 for 30 min. F-actin filaments were stained using Alexafluor phalloidin (Invitrogen, Calsbad, CA, USA) for 30 min. Nuclei were stained with a buffer of Propidium Iodide and RNase (BD Biosciences Pharmigen, San Diego, CA, USA) at 4 °C for 15 min and washed with PBS.

Statistical analysis was conducted using a descriptive analysis based in the amplitude and spatial parameters of the roughness. Results were compared before and after sintering at $\alpha$=0.05, using two-tailed Student's t-test or Mann–Whitney test, depending of the normality of distribution according to Kolmogorov-Smirnov test.

## 3. RESULTS AND DISCUSSION

Results are shown in two different ways: In the first approach, the microstamping process of silica was evaluated through SEM analysis of the PDMS micromolds and micropatterned silica coatings, and AFM imaging to quantify the contraction post-sintering. In the second approach, the biological behavior was evaluated in terms of adhesion and propagation of HBMSCs on silica micropatterns.

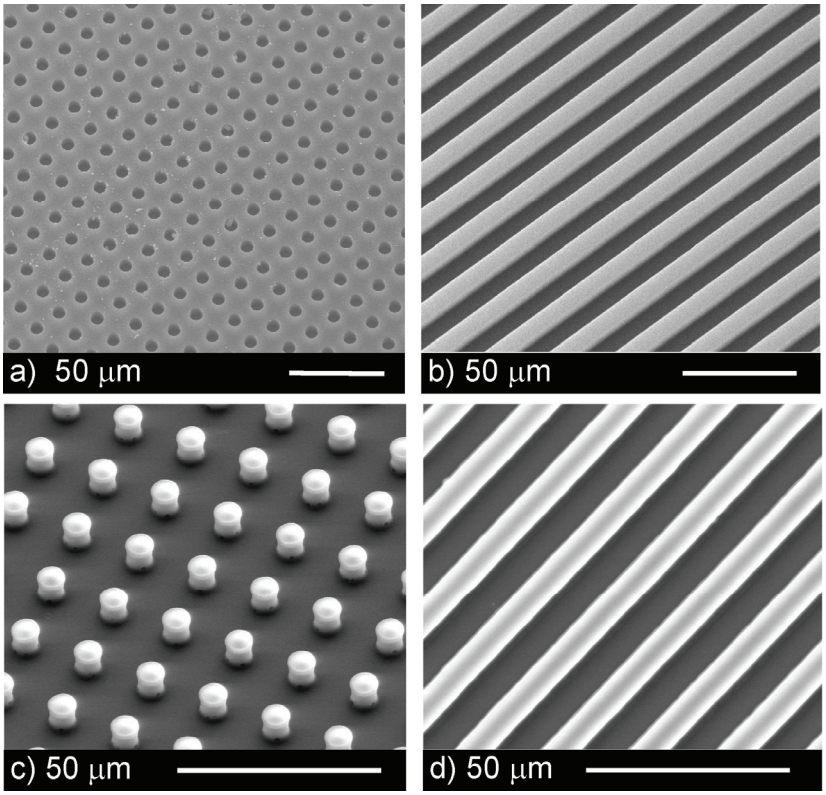



Figure 13. SEM images of PDMS molds (a,b) and silica micropatterned coatings (c,d), anisotropic (b,d) and isotropic (a,c) features with 5 μm spacing.

Examples of the PDMS molds and representative silica micropatterned coatings are shown in Figure 13. PDMS molds were made to produce isotropic and anisotropic microfeatures. Low and high magnification SEM images confirmed the capability of the soft lithography process to produce large, defect-free microstructured areas. This is an important pre-requisite to

obtain successful microstamped silica coatings (Marzolin, Smith *et al.*, 1998). The microstamping process produced micropatterned silica coatings with different microfeatures, as shown in Figure 13. However, changes in the dimension could affect the reproducibility of the process. Therefore, specific micromolding conditions should be tailored for each case to prevent defects such as collapsed microfeatures or delamination.

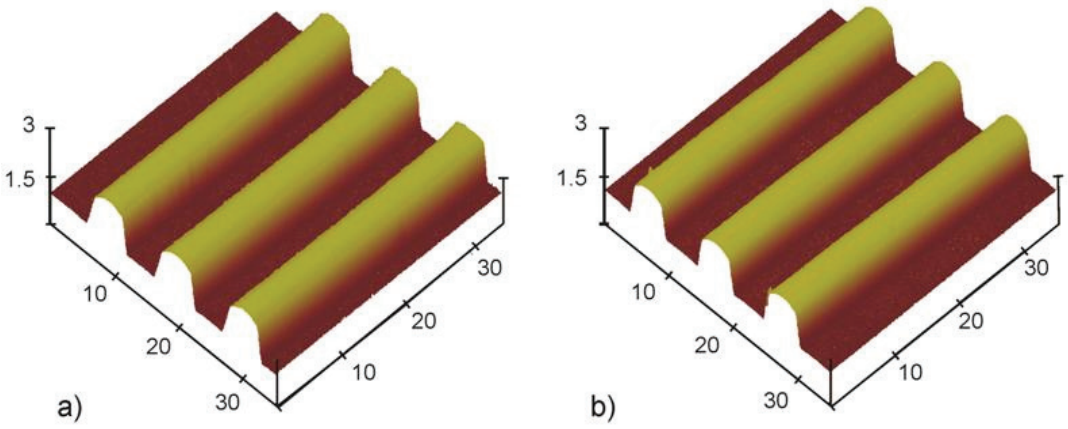



Figure 14. Silica micropatterned film with 5 µm wide features with 5 µm spacing. a) Non-sintered and b) sintered for 30 min at 500 °C. X,Y,Z axis are in micrometers.

AFM images (Figure 14) obtained before and after sintering, did not reveal statistical significant ($p \leq 0.05$) dimensional changes (Table 6). The post-sintering contraction calculated along Z axis (normal to the sample surface) was 10.9%. In the present study, a non-destructive AFM-based evaluation was conducted to quantify the contraction of the micropatterns after sintering. This approach uses a self-controlled image of the same specimen for comparison purposes. This concept was used previously (Batista, Junior *et al.*, 2007) to evaluate biological specimens before and after chemical and physical treatment in dentistry. Other techniques, such as SEM and TEM, require multiple specimens, and in the specific case of silica produced by sol-gel, deterioration can occur due to thermally-induced stresses (Gallardo, Galliano *et al.*, 2000b) from interactions with the electron beam.

Table 6 showed the results of roughness in terms of amplitude and spatial parameters. For amplitude parameters, *Ra and Rq* were higher in the sintered surface than in the non-sintered surface. For parameters related with the distribution of amplitude as *Ssk* and *Sku,* lower values were found for the sintered group than for the non-sintered group. However, the differences were not statistically significant ($p>0.05$).

**TABLE 6. ROUGHNESS ANALYSIS OF AMPLITUDE AND SPATIAL PARAMETERS BEFORE AND AFTER SINTERING OF MICROPATTERNED SILICA COATINGS**

| | Parameters | | Units | Non-sintered | | Sintered | |
|---|---|---|---|---|---|---|---|
| | | | | Mean | SE | Mean | SE |
| Amplitude | Roughness Average | Sa | nm | 14.34 | 2.27 | 17.50 | 3.49 |
| | Root Mean Square | Sq | nm | 20.20 | 2.60 | 22.78 | 4.08 |
| | Surface Skewness | Ssk | | 1.24 | 0.85 | 0.38 | 0.49 |
| | Surface Kurtosis | Sku | | 40.75 | 19.33 | 15.01 | 5.61 |
| Spatial | Density of Summits | Sds | 1/um² | 2.75 | 0.14 | 2.98 | 0.07 |
| | Texture Direction Index | Stdi * | | 0.23* | 0.01 | 0.33* | 0.00 |
| | Dominant Radial Wave Length | Srw | µm | 27.06 | 4.58 | 27.16 | 4.52 |
| | Radial Wave Index | Srwi | | 0.10 | 0.00 | 0.11 | 0.00 |
| | Mean Half Wavelength | Shw | µm | 1.81 | 0.14 | 1.8 | 0.19 |
| | Fractal Dimension | Sfd | | 2.67 | 0.05 | 2.72 | 0.01 |
| | Correlation Length at 20% | Scl20 | µm | 2.19 | 0.07 | 2.28 | 0.02 |
| | Texture Aspect Ratio at 20% | Str20 | | 0.13 | 0.00 | 0.13 | 0.00 |
| | Correlation Length at 37% | Scl37 | µm | 1.80 | 0.06 | 1.87 | 0.02 |
| | Texture Aspect Ratio at 37% | Str37 | | 0.10 | 0.00 | 0.11 | 0.00 |

* Statistically significant differences ($p\leq0.05$)

The sintering process produced changes on the roughness, starting in values of *Ssk>1* that may indicate the presences of considerable holes or peaks on the surface in the non-sintered state, to *Ssk>0* that may indicate the presence of a flat surface with peaks. A reduction in the *Sku* parameter may indicate a broaderly uniform height distribution. For the spatial parameters, a trend was observed towards reducing or maintaining equal all the parameters after sintering, with the exception of *Stdi* parameter. All differences found between both sintering stages were not statistically significant for all the calculated parameters, except for the *Stdi* parameter that showed an increase. When *Stdi* show values close to zero it may indicate that texture has a very dominant direction, while values close to one indicate that no dominant direction exists.

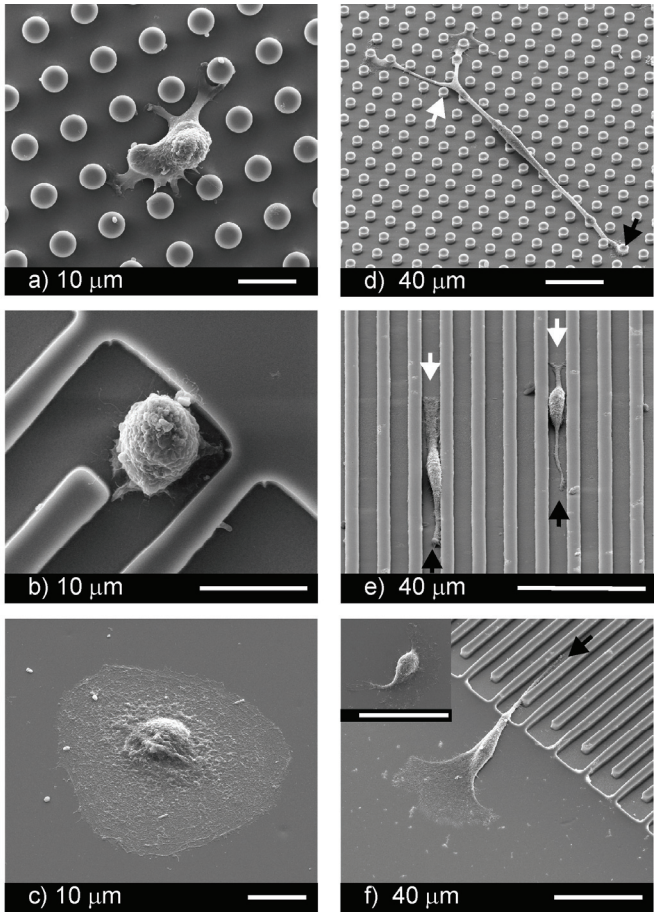



Figure 15. Adhesion and early spreading at 4h (a-c) and 24h (d-f).

SEM Images (SE mode) of 5 μm micropatterned Pillars and Lines and flat silica coatings. The white arrows indicate the leading edges of the cells, with the trailing edges indicated with black arrows.

Adhesion and early spreading of HBMSCs is shown in Figure 15 At the 4h time point, three different stages of adhesion/spreading may be clearly observed in the different groups. Initial cell-material contact with presence of filopodia may be observed in the lines group (Figure 15b). While in the pillars group a initial spreading (Figure 15a) was observed, where a cell on top of two adjacent pillars with an immature lamellipodia-like formation extending to the bottom of the pillars was observed. An intermediate spreading (Figure 15c) can be seen on the flat surface. During this period, cells presented a radial spreading without forming initial cytoplasmic extensions.

At the 24h time point, a significant elongation could be observed for the cells on micropillars (Figure 15d). The cells are clearly polarized, growing on the top of pillars, and starting from the bifurcation (Figure 15d, white arrow), both projections exhibit alignment with the subjacent microstructure, while the contra-lateral pole presents a contact with the bottom surface (Figure 15d, black arrow). However, a clear direction of elongation could not be defined.

Cells on line-shaped micropatterns showed a clear polarization with the presence of cytoplasmatic projections. An important morphological difference between the cell poles was observed, one of the projections (Figure 15e, white arrow) presented a wide appearance, which is compatible with the morphology of a clearly defined lamellipodium at leading edge of a migrating cell. The contra-lateral pole presented a narrower cylindrical extension that resembles the trailing edge of the cells (Figure 15e, black arrow). In all the cases, the motion appeared to be along the direction of the micropaterned lines.

Cells growing at the border between a flat and micropatterned regions (Figure 15f) showed a different behavior. While a multidirectional spreading may be noted in the lamellipodia on the flat surface, a slimmer cytoplasmic extension as a tail of retraction is observed at the micropatterned side. The inset in Figure 15f shows a magnified image of a cell on a flat coating showing semi-radial spreading.

At the 7d time point, the cells exhibited a highly spread and flattened morphology, which decreased the contrast in the secondary electron (SE) mode of the SEM. Much clearer images could be obtained in backscattered electron (BE) mode, as this mode is based on compositional differences, helping in the detection of cells spreading on flat (Figure 16c) and microstructured coatings (Figure 16a-b). Marked differences were found, depending on the surface topography. Cells on flat surfaces showed an increased number of cells, area coverage and random alignment. On the other hand, cells growing on micropillar surfaces presented alignment and elongation, as was observed in the 24h culture (Figure 15d-e).

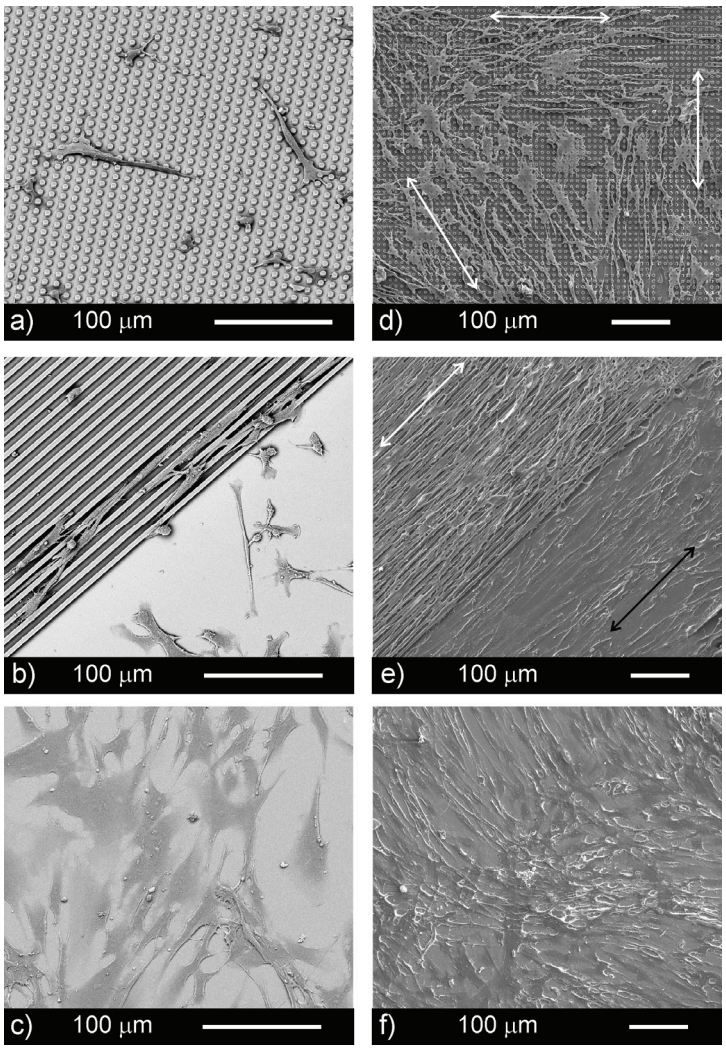



Figure 16. Proliferation and alignment on 5 μm micropatterned pillars (a,d), lines (b, e) and flat (c, f) silica coatings.

SEM images using BE mode (a-c) after 7d cell culture and using SE mode at 14d (d-f). Bar Scale = 100 μm. Arrows indicate the alignment and direction of spreading and propagation.

Fluorescence microscopy (Figure 17) was used to confirm that a clear HBM cellular alignment was induced on line-shaped micropatterned surfaces at 24h of culture (Figure 17b). These effects were maintained until day 7 (Figure 17e) on both stained cellular structures (F-actin filaments in green and nucleus in red). However, effects in the cells alignment were less evident on the pillars-shape micropatterned and on flat surfaces where cell distribution presented a randomized aspect.

The interface between flat and micropatterned surfaces is shown in Figure 16b, where cells on the micropatterned region showed alignment and slimmer cytoplasms. One of the major disadvantage in using BE mode is the

inability to evaluate the presence of dorsal filopodia, a structure that, when present, may be used to confirm cell health and that an adequate drying process has been conducted (Bohil, Robertson *et al.*, 2006).

At day14, an increase in HBM cells number on the micropillared surfaces was observed. A multi-directional alignment was seen on the isotropic surface (Figure 16d), where vertical, horizontal and oblique orientations could be clearly observed, relative to the underlying array of pillars. Figure 16e presents the interface between micropatterned and flat areas. The image shows that cell alignment was clearly induced by the microtextured surface (white arrows). This behavior was extended to the neighboring flat areas (black arrow), presumably as a result of further cell-cell interactions (e.g. release of soluble factor or through direct contact).

The concept of topographic control of cell behavior was introduced in 1983 in a study that showed that gingival primary cells grew in parallel with anisotropic grooves produced by mask-etching on titanium coatings (Brunette, Kenner *et al.*, 1983). Many studies have since been designed to evaluate different aspects of this concept, such as processing techniques, surface chemistry, surface energy, morphology and orientation at the micro-nano scale for 2D surfaces used for fundamental cellular studies or 3D surfaces produced in polymers to be used as scaffolds (Mata, Kim *et al.*, 2009; Kim, Boehm *et al.*, 2010). As it is well established, anisotropic textures resulted in alignment when the grooves presented an adequate height. Isotropic microfeatures did not induce cell alignment but they may promote other functions (Rompen, Domken *et al.*, 2006; Lim, 2009).

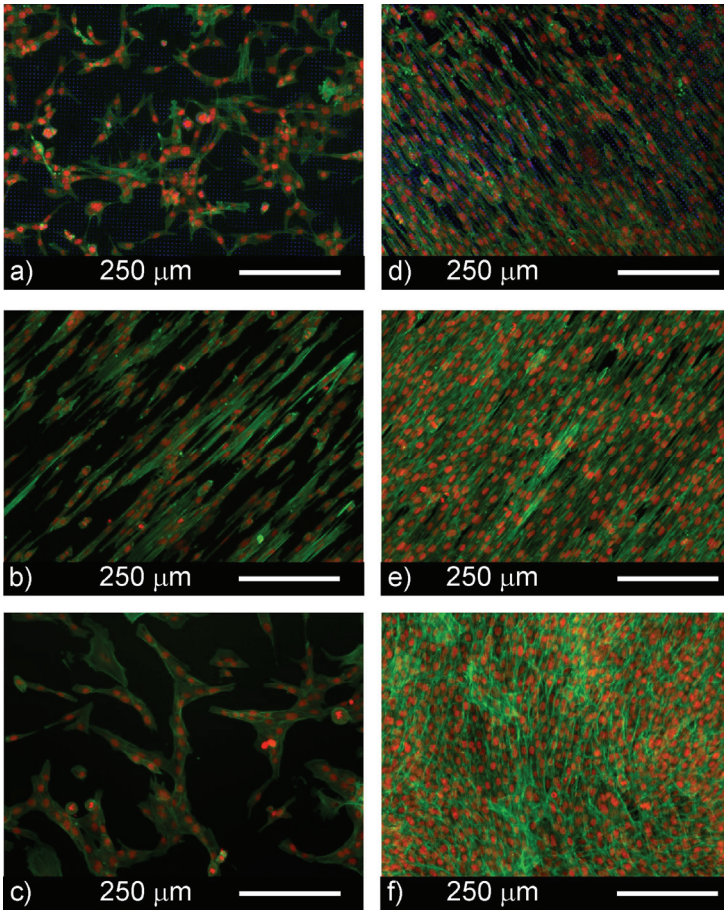



Figure 17. Fluorescence images of HBM cells cultured on micropatterned pillars (a,d), lines (b, e) and flat (c, f) silica coatings. Fluorescent labeling of F-actin (green) and DNA (red). Bar Scale = 250 μm.

Alteration in cellular response is not an exclusive property of organized textures, given that enhance adhesion, proliferation and differentiation have also been shown, using randomly textured surfaces produced by different techniques (i.e. sandblasting, grinding and electropolishing) (Curtis and Wilkinson, 1997).

The fundamental knowledge available on cell responses to organized textures has been based on evidence about surface modifications of polymers and metals, using epithelial cells, osteoblast, fibroblasts and neurons (Brunette, Kenner *et al.*, 1983; Ito, 1999; Falconnet, Csucs *et al.*, 2006; Kim, Boehm *et al.*, 2010). However the behavior of bone cells on microfabricated hard surfaces is still an open field.

## 4. CONCLUSIONS

From the SEM and AFM studies described in this work, it may be concluded that: (1) a combined technique based on soft lithography and sol-gel chemistry allowed us to produce patterned silica thin films with isotropic and anisotropic microtopographies and (2) that silica films microtopographies influenced cells response in terms of adhesion, spreading, cytoplasmic protrusions and migration.

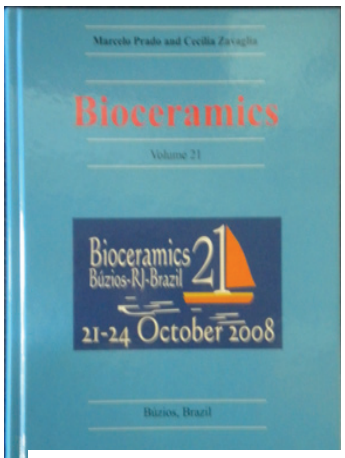
## 5. ACKNOWLEDGMENTS

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## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

# Manuscript 6. CELLULAR ALIGNMENT INDUCTION DURING EARLY IN VITRO CULTURE STAGES USING MICROPATTERNED GLASS COATINGS PRODUCED BY SOL-GEL PROCESS




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**Cellular alignment induction during early in vitro culture stages using micropatterned glass coatings produced by sol-gel process**

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# CELLULAR ALIGNMENT INDUCTION DURING EARLY *IN VITRO* CULTURE STAGES USING MICROPATTERNED GLASS COATINGS PRODUCED BY SOL-GEL PROCESS

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## ABSTRACT

Cell behavior such as adhesion, morphological features, proliferation and functional activity are highly influenced by surface properties including hydrophilicity, roughness, texture and morphology. These surface properties may be controlled using a mixture of additive coating techniques to produce glass coatings by sol-gel process and soft lithography on dental ceramics. The purpose of this work was to compare cell adhesion and early orientation of Human Bone Marrow (HBM) cells cultured on micro-patterned (micro-PGC) and on flat glass coatings (FGC) produced by sol-gel processing. Spin Coating was used to apply $SiO_2$ flat coatings on glass substrates as model surfaces. Photolithography was applied to produce master patterns within microscale dimensions. A moulding was used to print $SiO_2$ glass coatings produced by a sol-gel process. The coatings were then sintered, sterilized and cultured with HBM cells derived from primary cultures, using a standardized protocol, for 1 and 7 days. Cell morphology and orientation were followed using SEM and Confocal Laser Scanning Microscopy. Flat and MPGC with line shaped features were produced. Cells presented a

typical osteoblastic morphology on flat surfaces while slimmer, preferentially oriented and more elongated morphologies could be seen on line micro-patterned surfaces. HBM cells cultured on flat glass coatings showed increased tendency to spread and to assume more randomized proliferation when compared to the cells on the micro-patterned glass coatings. Micro-patterned glass coatings showed higher orientation control and smaller delay in the rate of proliferation, in early stages of *in vitro* culture than flat coatings. These preliminary studies revealed that micro-PGC induce significant morphological changes and controlled orientation of HBM cells during early stages of cell proliferation.

## 1. INTRODUCTION

Some titanium implants do not integrate, do not survive for a long enough time, and may be affected by peri-implantitis or have poor aesthetics (Warashina, Sakano *et al.,* 2003; Scarano, Piattelli *et al.,* 2004). Recent developments in dental ceramics have introduced Ytria stabilized Zirconia as a ceramic material with high mechanical strength, better aesthetic performance due its colour closer to natural teeth's (Manicone, Rossi Iommetti *et al.,* 2007) and without any reported cytotoxic or mutagenic effects (Covacci, Bruzzese *et al.,* 1999; Silva, Lameiras *et al.,* 2002). However, this material presents reduced bioactivity. Furthermore, there is a lack of information regarding long-term clinical success rates for dental applications (Oliva, Oliva *et al.,* 2007).

Peri-implantitis is generally defined as a chronic inflammation of the support tissues around the implant produced by bacterial colonization, facilitated by the implant/abutment gap, chemical composition and/or surface roughness of the restorative components (Klinge, Hultin *et al.,* 2005).

Low bacterial adherence and osteointegration may be obtained with a microfabrication strategy such as soft lithography and bioactive glass based nanostructured coatings by sol-gel process onto ceramic components like the abutments and screws. Surface texture of dental implants affects the rate of osteointegration and biomechanical fixation (Wennerberg, Albrektsson *et al.*, 1995).

Glass coatings applied by sol-gel process (Pelaez, Pareja *et al.*, 2005) and soft lithography on ceramics have produce surfaces where control of chemistry, roughness, thickness and textures are possible (Curtis and Wilkinson, 1997; Ito, 1999; Tan and Saltzman, 2004). In this work, the association of microfabrication techniques and sol-gel processing was used to produce specifically controlled surfaces for topographical modulation studies on cell spreading on ceramic biomaterials.

During the first half of the last century it was discovered that cell behavior was affected by the topographical morphology of the surfaces. This phenomenon was first named as stereotropism and later became known as contact guidance. Brunette was among the first to propose the use of micro-grooved implant surfaces to prevent apical epithelial migrations (Brunette, Kenner *et al.*, 1983).

The purpose of this work was to compare early orientation of Human Bone Marrow (HBM) cells on micro-patterned (micro-PGC) and on flat glass coatings (FGC) produced by sol-gel processing.

## 2. MATERIALS AND METHODS

The sol-gel process was used to produce $SiO_2$ flat and micropatterned glass coatings using hybrid silica sols resulting from acid catalysis in a single

stage. Tetraethylorthosilicate (TEOS, Aldrich) and methyltriethoxisilane (MTES, Aldrich) were selected as silica precursors for the sol, while alcohol was used as a solvent and nitric acid and acetic acid were used as catalysts. Spin Coating technique was chosen to apply $SiO_2$ flat coatings on microscope glass slides as model substrates.

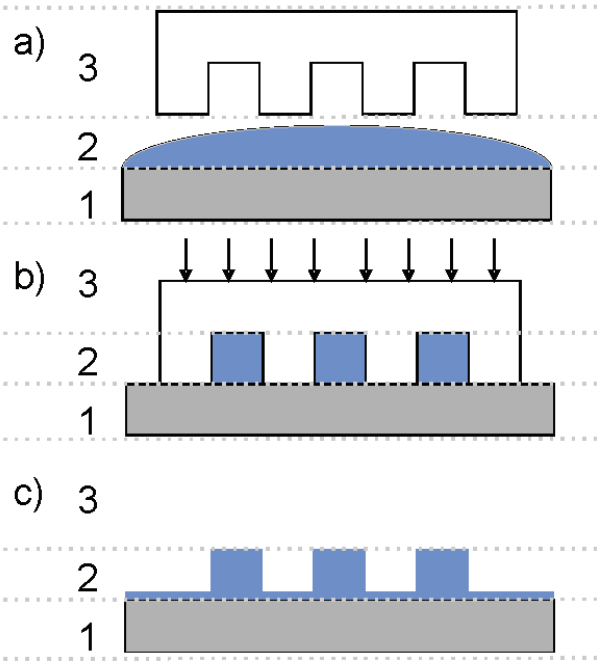



Figure 18. Micromoulding process:

a) Preparation of substrate (1) molten drop of $SiO_2$ solution prepared by Sol-Gel process (2) and PDMS micromolding template (3) produced by soft lythography. b) Pressed with constant pressure and aged for 2 hours and c) demoulding and sintering.

Photolithography was used to produce master patterns with microscale dimensions. A polydimethylsiloxane (PDMS) moulding (Figure 18) was obtained from the masters by uniformly mixing PDMS (Silastic T-2, Dow Corning, USA) with a curing agent, pouring the mixture onto the masters, degassing, and curing (Gallego, Ferrell *et al.*, 2008). The coatings were sintered using a thermal cycle that included heating up to 450ºC at a rate of 20ºC/min, a plateau at 450ºC for 30 min and cooling in air using an elevator furnace, followed by autoclave sterilization.

Human Bone Marrow (HBM) was obtained from patients undergoing orthopaedic surgery procedures. Informed consent to use this biological material, that would be otherwise discarded, was obtained. Bone marrow was cultured in α-minimal essential medium ($\alpha$-MEM) containing 10% fetal bovine serum, penicillin-streptomicine (100 IU/ml and 10 mg/ml, respectively), fungizone (2.5 µg/ml) and ascorbic acid (50 µg/ml). Primary cultures were incubated in a humidified atmosphere of 5% $CO_2$ in air at 37 °C, and the medium was changed twice a week. Primary cultures were maintained for 10/15 days till near confluence, when adherent cells were enzymatically released with 0.04% trypsin and 0.025% collagenase.

Coated samples with and without micro-patterns were cultured with HBM cells ($3^{th}$ and $5^{th}$ passage), under the same conditions of Primary cultures for 1 and 7 days. Cell morphology and orientation were followed using SEM and Confocal Laser Scanning Microscopy (CLSM) according to the following sequence: Three cultured samples per group were washed with PBS, fixed using 1.5% glutaraldehide, dehydrated in graded alcohol solutions, gold sputtered and observed by SEM. For CLSM, three samples per group were washed with PBS, fixed in 4% v/v formaldehyde (methanol-free; Polyscience) for 15 min, permeabilized with 0.1% v/v Triton X-100 for 5 min, and incubated in 10 mg/ml bovine serum albumin and 100 µg/ml RNAse for 45 min at room temperature. F-actin filaments were stained with Alexafluor-conjugated phalloidin (Molecular Probes) for 20 min and nuclei were counterstained with 10 µg/ml propidium iodide (Sigma) for 10 min.

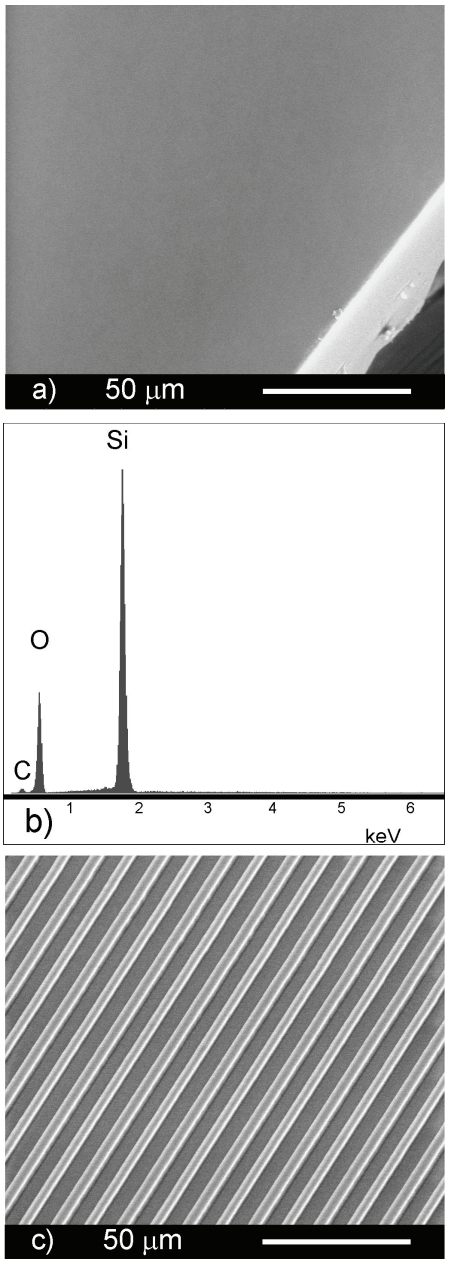



Figure 19. Silica Coatings.

a) Flat $SiO_2$ coating, in the lower-right corner, the boarder of the film applied by Spin Coating is shown, b) representative EDS analysis of both types of $SiO_2$ coatings and c) Micro-patterned $SiO_2$ coating.

Finally, samples were washed with PBS and mounted and observed on a BioRad MRC 600 microscope and postprocessed with Leica Confocal Software (Leica, Mannheim, Germany).

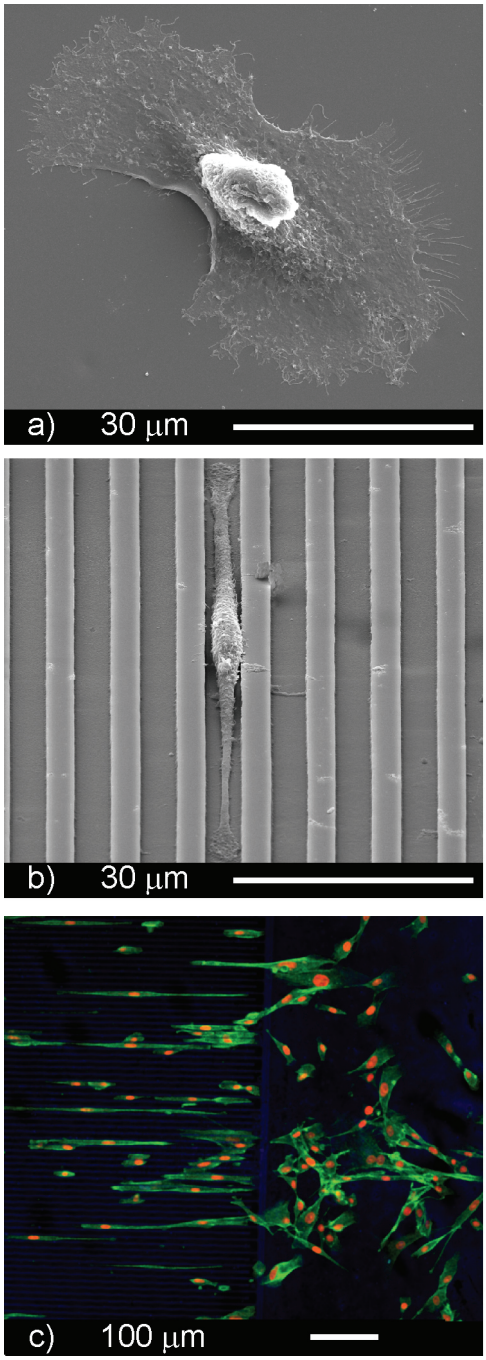



Figure 20. SEM and CLSM images of HBMSCs

SEM images of a) Flat $SiO_2$ coating and b) Micro-patterned $SiO_2$ coating with HBM cells cultured for 24h. c) CLSM image of interface between micropatterned (on the left side of the image) and flat coatings with HBM cells cultured for 7 days.

## 3. RESULTS AND DISCUSSIONS

Flat and MPGC with line shaped features with 5μm width in the top ridges and ~3μm height were produced (Figure 19). The EDS analyses showed $SiO_2$ on both types of surfaces Figure 19. Bone marrow cells were able to adhere and spread on both surfaces. Attached cells presented a spread –

polygonal shape with cytoplasmic extensions adapting to the flat surface while slimmer, preferentially oriented and more elongated morphologies could be seen on line micro-patterned surfaces. HBM cells cultured on flat glass coatings showed increased tendency to spreading and to assume more random proliferation distributions when compared to the cells on the micro-patterned glass coatings. HBM cells on micro-patterned glass coatings showed alignment parallel to long axis of ridges and smaller delay in the rate of proliferation in 7$^{th}$ day of culture than flat coatings (Figure 20). These results are in agreement with other previously reported works (Brunette, Kenner *et al.,* 1983).

## 4. CONCLUSIONS

These preliminary studies revealed that micro-PGC induce significant morphological changes and controlled orientation of HBMSCs during early stages of cell proliferation.

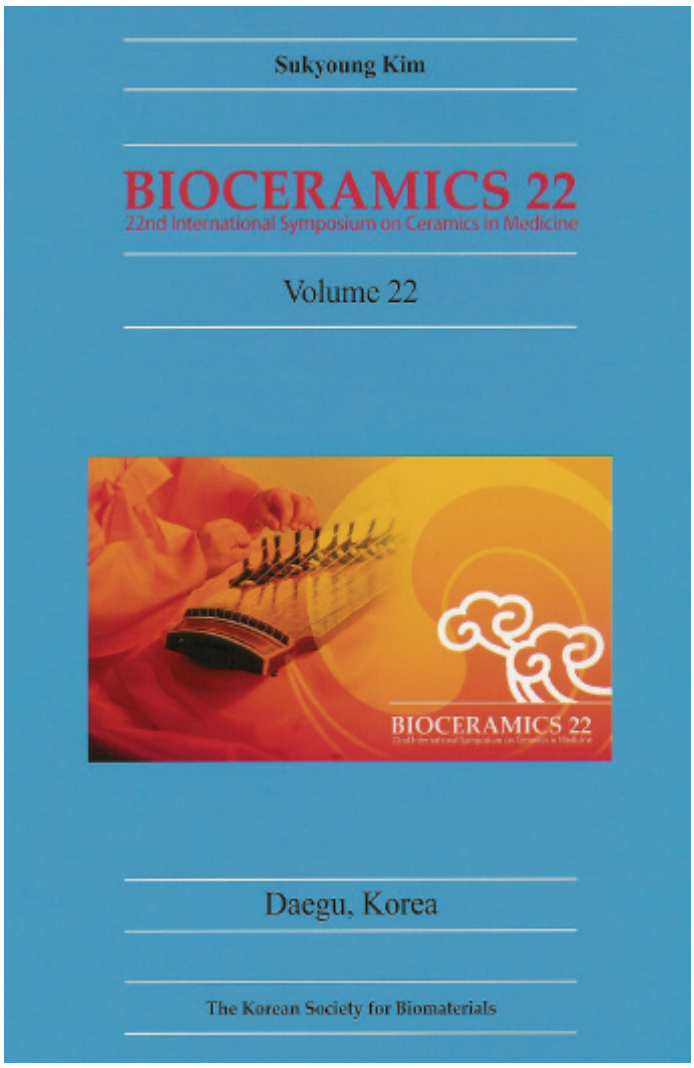
## 5. ACKNOWLEDGMENTS

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## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

# Manuscript 7. MICROFABRICATED $SIO_2$ THIN FILMS ON STRUCTURAL DENTAL CERAMICS




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# MICROFABRICATED $SIO_2$ THIN FILMS ON STRUCTURAL DENTAL CERAMICS

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## ABSTRACT

The purpose of this study was to develop a technique to obtain microstructured $SiO_2$ coatings on 3Y-TZP based on soft lithography micropatterning and sol-gel processing.

3Y-TZP commercial powders and green samples were characterized by SEM/EDS, DSP and dilatometry analysis. 3Y-TZP disks were processed and examined by XRD and SEM. PDMS molds with various microfeature geometries were used to microfabricate sol-gel processed $SiO_2$ thin films on Y-TZP substrates. Preliminary cytocompatibility studies were conducted with human osteoblast-like cell cultures using a standardized protocol.

DSP analysis of 3Y-TZP raw powders showed a bimodal distribution. The highest contraction was found at 1300˚C. The micropatterning technique implemented for the $SiO_2$ thin films allowed the production of features with lateral dimensions as small as ~1µm over large areas. Induced cellular alignment was observed during early culture stages on the micropatterned

glass coatings.. In conclusion, soft lithography and sol-gel chemistry were combined to produce a reliable technique to micropattern $SiO_2$ thin films on structural dental ceramics. This technique could be used to modify surfaces in a controlled manner to modulate the biological response and enhance the success rate of ceramic dental implants.

## 1. INTRODUCTION

The history of biomaterials research for dental implants can be analyzed from two different approaches: the pragmatic, based in clinical success; and the theoretical, based in materials science research. However, it is well accepted that contemporary implant history started with the work by Branemark's group on titanium osseointegration (Albrektsson, Sennerby *et al.*, 2008). From a clinical perspective, the success of titanium implants is due to their osseointegration. Other materials such as zirconia, alumina, tantalum and niobidium also exhibit this property, but are not widely commercialized (Johansson, Hansson *et al.*, 1990; De Wijs, Van Dongen *et al.*, 1994; Oliva, Oliva *et al.*, 2007).

Ceramics for intra-osseous dental implants have been successfully used to mimic the appearance of natural teeth, but the main drawbacks are their mechanical properties (De Wijs, Van Dongen *et al.*, 1994). Since the introduction of yttria-stabilized Zirconia (Y-TZP) for dental applications, an apparent answer for the poor mechanical properties was obtained (Denry and Kelly, 2008); however, previous studies on low temperature degradation show elution of yttria and transformation of the tetragonal phase in wet environments (Chevalier, 2006). Nevertheless, clinicians are showing increasing interest in Y-TZP as rehabilitation dental material (Denry and Kelly, 2008).

3Y-TZP requires precise ceramic processing to obtain suitable materials for applications in clinical dentistry. Topics like synthesis of raw powders, molding, sintering, milling and polishing have been extensively studied (De Aza, Chevalier *et al.,* 2002), but reports about surface modification to improve the biological response are limited (Griggs, Kishen *et al.,* 2003). Currently, the techniques developed for surface modification of titanium dental implant cannot be applied for all-ceramic implants. Our hypothesis is that sol-gel processing is a viable alternative to modify Y-TZP surfaces due its versatility to produce powders, coatings and bulk biomaterials.

The purpose of this study was to develop a technique to obtain microstructured $SiO_2$ coatings based in soft lithography micropatterning and sol-gel processing to obtain early guided cellular interaction on Y-TZP.

## 2. MATERIALS AND METHODS

Zirconia disks were used as substrates to test the microstructured $SiO_2$ thin films. 3Y-TZP powders (Tosho, JP) were characterized to confirm the information provided by the manufacturer. Particle size distribution was determined via laser diffraction analysis (Mastersizer S, U.K.). Raw powders were uniaxially pressed (150 MPa) and the sintering behavior was evaluated by dilatometry (Setsys 16/18, Setaram, France) at a heating rate of 5˚C/min from 20˚C to 1550˚C. Taking into account dilatometric data, samples were sintered in air atmosphere at 1480˚/h using a heating rate of 5˚C/min to obtain dense compacts. The bulk densities of the sintered bodies were determined by water displacement method (Arquimedes Method). The crystalline phases of the sintered samples were established by XRD analysis (Siemens D5000, Germany).

$SiO_2$ sol was produced via acid catalysis in a single step using Tetraethylorthosilicate (TEOS, Aldrich) and Methyltriethoxisilane (MTES, Aldrich) as precursors following a previously reported method (Pelaez, Garcia *et al.*, 2004; Pelaez, Pareja *et al.*, 2005; Pelaez-Vargas, Ferrell *et al.*, 2009b). The solution was aged for 24h and applied on 3Y-TZP substrates to produce $SiO_2$ flat coatings using spin coating (3000 rpm at 45s).

A silicon master was obtained using a chromium mask with different features via standard UV photolithography. The master was used to produce a PDMS (Polydimethylsiloxane) mold. 3Y-TZP substrates were cleaned and dried. The sol was poured (40 µl) on the PDMS mold and the substrate placed on top of the sol. After drying (2h) the mold was removed (Marzolin, Smith *et al.*, 1998), the sample evaluated under light microscopy, and then sintered at 500˚C for 60min.

MG63 human osteoblast-like cells (ATCC, USA) were cultured in α-Minimal Essential Medium (α-MEM) containing 10% fetal bovine serum, penicillin-streptomycin (100 IU/ml and 10 mg/ml, respectively), gentamicin-amphotericin B (10 µg/ml and 0.25 µg/ml) and ascorbic acid (50 µg/ml). The cells were seeded at a density of $2x10^4$ cells/$cm^2$ on micropatterned and flat $SiO_2$ coatings. All cultures were incubated for 24h. Cells were washed with warm PBS, fixed in 2.5% glutaraldehyde, dehydrated/dried in graded ethanol and hexamethyldisilazane (HMDS, Ted Pella, USA) solutions (50%-100%) (Braet, De Zanger *et al.*, 1997), gold coated and visualized via SEM (HITACHI, S-3000H).

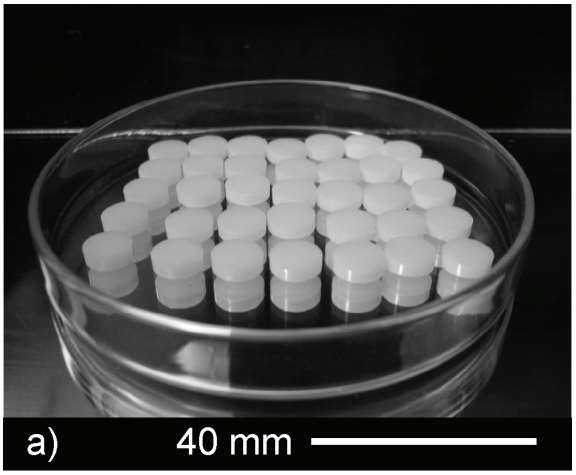
## 3. RESULTS AND DISCUSSION

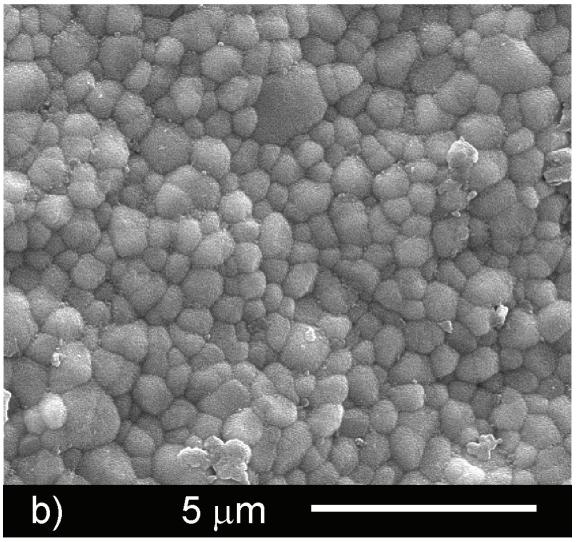
DSP analysis of 3Y-TZP raw powders showed a bimodal-like distribution ($D_{50}$= 0.38µm). The dilatometry showed that the highest

contraction rate was obtained at 1300˚C; however the optimum sintering temperature to ensure high densification and full transformation of zirconia from monoclinical to tetragonal phase was established at 1480˚C, which was further confirmed by XRD analysis (data not shown). The fabricated 3Y-TZP disks (Figure 21a) exhibited an apparent density close to the X-ray theoretical density value of t-$ZrO_2$ (6.07 g/$cm^3$) (Argyriou and Howard, 1995).

The micropatterning technique implemented for the $SiO_2$ thin films allowed for the production of a number of feature geometries with different lateral dimensions. Figure 21c shows the resolution array used to evaluate the reproducibility of the technique with different geometries and/or scales through single observation experiments. Successful micropatterning of structures as small as ~1 μm was obtained over large areas.

An array of 5 μm diameter pillars spaced by 5 μm (edge to edge) was chosen as model geometry to study the interactions between the cells and the microfabricated $SiO_2$ thin films. Figure 22b-c show cells at different adhesion and spreading phases. Induced cellular alignment was observed during early culture stages on the micropillar-patterned $SiO_2$ coatings on Y-TZP. This could find applications in guided tissue regeneration in dentistry. Figure 22d shows a cell in multidirectional spreading on a flat $SiO_2$ coating. Polishing marks on the Y-TZP surface are observed under the $SiO_2$ thin film.



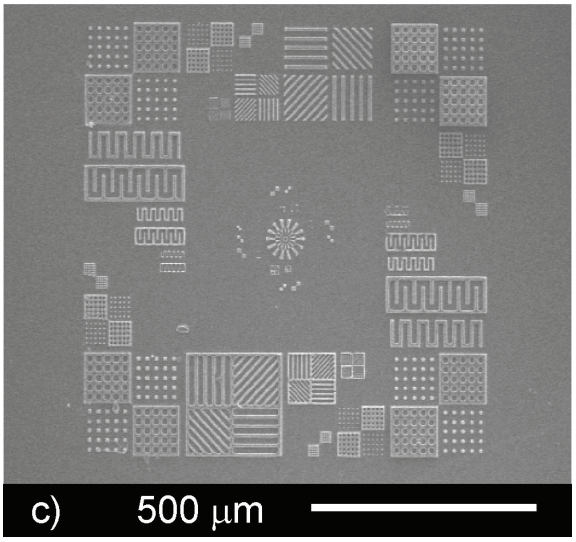




Figure 21. Substrates and $SiO_2$ coatings.

a) 3Y-TZP disc substrates. b) SEM image of the 3Y-TZP disc surface, the narrow range of variation in grain size (~1μm) could be related with the high apparent density. c) Micrograph showing an array of $SiO_2$ features with different geometries. Bars (a= 40mm, b=5μm, c=500μm)

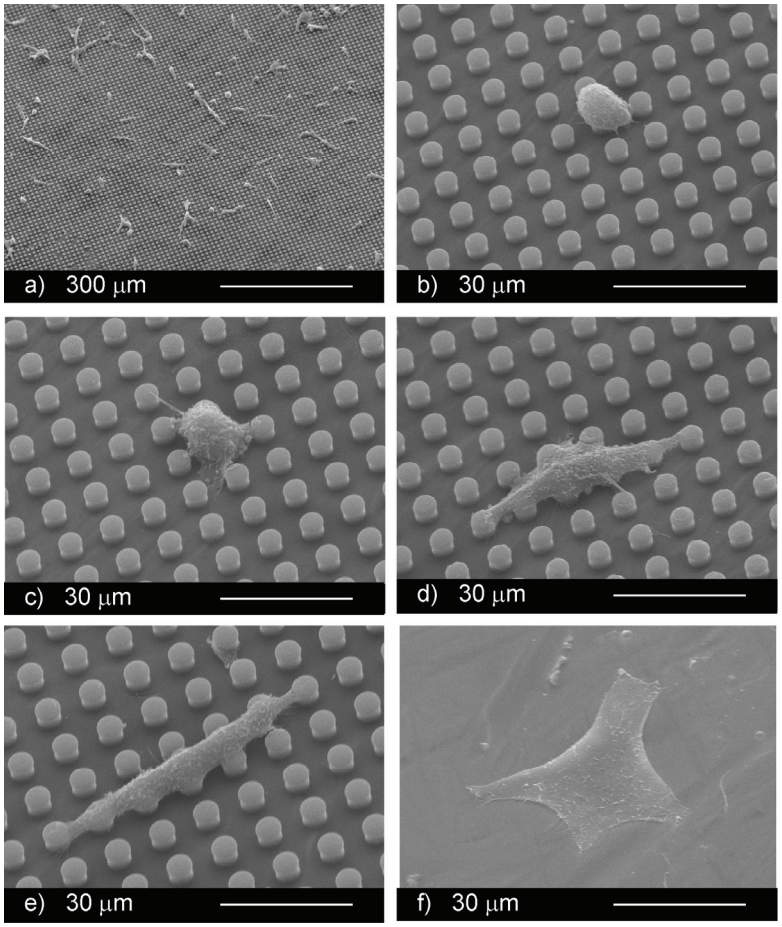



Figure 22. Early adhesion (24h) and spreading of MG63 cells on micropatterned $SiO_2$ pillars applied on Y-TZP substrates.

a) Low magnification image of the cells on the micropatterned surface (bar 300 μm). b) Rounded cell in initial contact with the surface. c) Cell showing initial adhesion trough filopodia. d) and e) show medium and high cellular alignment on the top of the pillars, respectively. f) Cell in multidirectional spreading on a flat $SiO_2$ coating. Bars (b-f =30 μm).

## 4. CONCLUSIONS

Soft lithography and sol-gel chemistry were combined to produce a reliable technique to micropattern $SiO_2$ thin films on structural dental ceramics. This technique could be used to modify surfaces in a controlled manner to modulate the biological response and enhance the success rate of ceramic dental implants.

## 5. ACKNOWLEDGMENTS

The authors acknowledge the support of the *Fundação para a Ciência e a Tecnologia* (FCT/SFRH/BD/36220/2007), CRUP-*Acções Integradas Luso-Espanholas (*46/09*),* Acciones Integradas *Hispano-Portuguesas* (HP2008-0075), and AFOSR MURI.

## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

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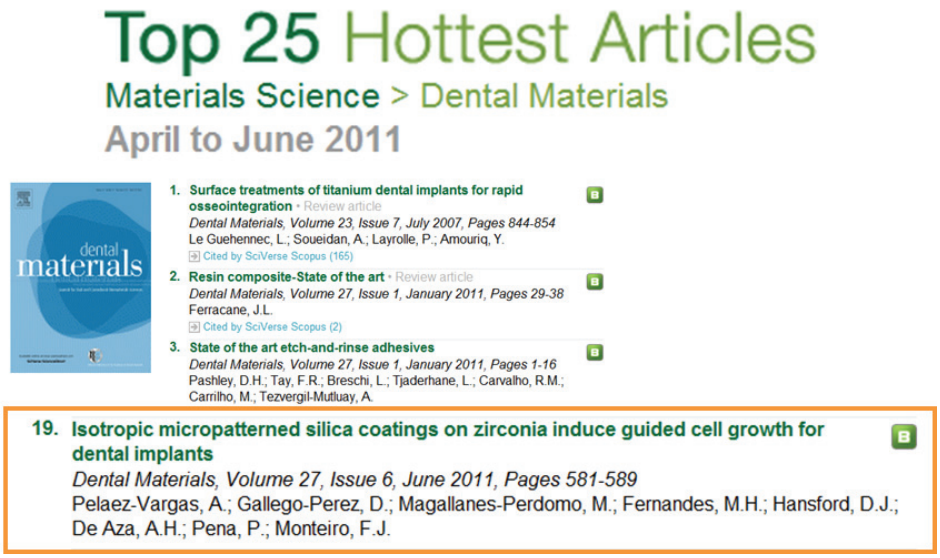
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**Isotropic micropatterned silica coatings on zirconia induce guided cell growth for dental implants**

Published in Dental Materials. 2011;27(1):581-9.




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# ISOTROPIC MICROPATTERNED SILICA COATINGS ON ZIRCONIA INDUCE GUIDED CELL GROWTH FOR DENTAL IMPLANTS

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## ABSTRACT

Titanium implants are the gold standard in dentistry; however, problems such as gingival tarnishing and peri-implantitis have been reported. For zirconia to become a competitive alternative dental implant material, surface modification techniques that induce guided tissue growth must be developed. Objectives: To develop alternative surface modification techniques to promote guided tissue regeneration on zirconia materials, for applications in dental implantology. Methods: A methodology that combined soft lithography and sol-gel chemistry was used to obtain isotropic micropatterned silica coatings on Yttrium Stabilized Zirconia substrates. The materials were characterized via chemical, structural, surface morphology approaches. In vitro biological behavior was evaluated in terms of early adhesion and viability/metabolic activity of human osteoblast like cells. Statistical analysis was conducted using one way Anova/Tukey HSD post hoc test. Results: Isotropic micropatterned silica coatings on Yttrium

Stabilized Zirconia substrates were obtained using a combined approach based on sol-gel technology and soft lithography. Micropatterned silica surfaces exhibited biocompatible behavior, and modulated cell responses (i.e. inducing early alignment of osteoblast like cells). After 7 days of culture, the cells fully covered the top surfaces of pillars microstructured silica films. Significance: The micropatterned silica films on zirconia showed a biocompatible response, and were capable of inducing guided osteoblastic cell adhesion, spreading and propagation. The results herein presented suggest that surface modified ceramic implants via soft lithography and sol gel chemistry could potentially be used to guide periodontal tissue regeneration, thus promoting tight tissue apposition, and avoiding gingival retraction and peri-implantitis.

Commercially pure titanium (Ti) is the biomaterial of choice for dental implants because it is a relatively inexpensive material, promotes osseointegration, has well-documented biocompatibility, self-passivation, strength, and conformability (Kasemo, 1983). However, it has also been reported that Ti could exhibit problems such as potential allergenic response and poor aesthetics (Tschernitschek, Borchers *et al.*, 2005; Egusa, Ko *et al.*, 2008). Since the introduction of titanium dental implants, many other materials have been explored as potential implant candidates, including zirconia, alumina, tantalum and niobium, but some have never been commercialized, and the others have achieved very limited commercial success (Albrektsson and Lekholm, 1989; Johansson, Hansson *et al.*, 1990; Albrektsson and Sennerby, 1991; De Wijs, Van Dongen *et al.*, 1994; Oliva, Oliva *et al.*, 2007; Albrektsson, Sennerby *et al.*, 2008; Sennerby, 2008).

Many patients present large challenges for clinicians, including those requiring implant-supported dental single crowns in the anterior segment and patients with highly demanding aesthetic conditions due to the presence

of high line upper lip, a thin gingival biotype, or intact natural adjacent teeth (Leblebicioglu, Rawal *et al.,* 2007). The most common solutions include titanium-based dental implants, metallic or ceramic abutments, and all ceramic crowns (e.g., alumina, zirconia or combination) (Chevalier, 2006).

Zirconia has been studied due to its potential use in dentistry as inlays, onlays, full crowns, bridges, abutments, intra-root posts, scaffolds and implants (Akagawa, Hosokawa *et al.,* 1998; Hochman and Zalkind, 1999; Griggs, Kishen *et al.,* 2003; Manicone, Rossi Iommetti *et al.,* 2007; Oliva, Oliva *et al.,* 2007; Kim, Shin *et al.,* 2008; Malmstrom, Slotte *et al.,* 2009). No cytotoxic or mutagenic effects have been reported, and it has been shown to exhibit osseointegration as well as low inflammatory infiltrate in gingival tissues (Covacci, Bruzzese *et al.,* 1999; Amaral, Costa *et al.,* 2002; Kohal and Klaus, 2004; Sennerby, Dasmah *et al.,* 2005; Degidi, Artese *et al.,* 2006; Manicone, Rossi Iommetti *et al.,* 2007; Kohal, Att *et al.,* 2008). However, only preliminary results have been published, and long term studies are not available. Therefore, further research is required to develop an effective and competitive zirconia-based material candidate as a clinical alternative to titanium implants. Since the initial reports in 1980, dental ceramic implants have shown superior aesthetic behavior, as an adequate appearance of natural teeth could be obtained; however, their mechanical properties have been questionable (De Wijs, Van Dongen *et al.,* 1994).

For orthopedic and dental applications, a reduction in the catastrophic failure of all-ceramic restorations was obtained with the introduction of Yttria-Stabilized Tetragonal Zirconia Polycrystalline (3Y-TZP) (Garvie, Hannink *et al.,* 1975; Denry and Kelly, 2008). However, based on investigations of the use of 3Y-TZP in orthopedics, under physiological conditions, yttrium may leach from the ceramic elution and consequently the zirconia may lose transformational toughening (its crystal structure changes back from

tetragonal to monoclinic phases, known as "aging") (Pascual and Duran, 1983; Argyriou and Howard, 1995; Palin, Fleming *et al.*, 2001; Ghosh, Suri *et al.*, 2006; Lughi and Clarke, 2007). After more than 20 years of research, aging still remains a key issue for zirconia implants, with aging of zirconia orthopedic devices (e.g. femoral heads) by far the most studied example. Aging occurs by a water-assisted martensitic phase transformation, which propagates at the surface by a nucleation-and-growth mechanism and then invades the bulk structure. Therefore, zirconia could be prone to aging in the presence of human saliva. Aging results in roughening and microcracking, and in the most extreme cases, it leads to catastrophic failure of the component (Chevalier, 2006). Considering that the use of 3Y-TZP as a dental material is still increasing (Denry and Kelly, 2008) and that its low temperature degradation is still latent, further extensive clinical research is necessary to achieve a risk reduction (Lughi and Sergo, 2010).

Advanced ceramic processing to obtain 3Y-TZP materials is a demanding process. Tailoring the microstructure by refining powder processing can result in zirconia dental structures at the top end of their strength spectrum (Chevalier, Aza *et al.*, 2000). However, since the introduction of 3Y-TZP as a material for dental implants, only a limited number of reports concerning surface modification strategies have been produced, and the know-how available from decades of surface modifications of titanium implants is not directly applicable (Griggs, Kishen *et al.*, 2003).

Currently, surface modification techniques are based on the removal of material through low energy processing such as etching (chemical or electrochemical), or mechanical techniques such as grinding, sandblasting or ablation by electrons or lasers (Kosmac, Oblak *et al.*, 1999; Lee, Lee *et al.*, 2005; Nayab, Jones *et al.*, 2005; Perrie, Rushton *et al.*, 2005; Teixeira, Monteiro *et al.*, 2007; Vorobyev and Guo, 2007). These alternatives, however, can lead to yttria

leaching, phase transformation and subsequent catastrophic failure. Alternative additive approaches can be costly, present adhesion problems, and introduce heterogeneities (Filiaggi, Pilliar *et al.*, 1996; Griggs, Kishen *et al.*, 2003; Thian, Huang *et al.*, 2006; Sollazzo, Palmieri *et al.*, 2008; Sollazzo, Pezzetti *et al.*, 2008).

This work explored the use of sol-gel processing as a viable approach to modify 3Y-TZP surfaces, based on its versatility to yield materials with high chemical homogeneity and fine grained or amorphous microstructure using lower temperatures, and its ease of implementation for mass production (Klein, 1985; Albrektsson and Lekholm, 1989; Albrektsson and Sennerby, 1991; McDonagh, Sheridan *et al.*, 1996; Hench, 1997; Garcia, Galliano *et al.*, 2003; Pelaez, Garcia *et al.*, 2004; Albrektsson, Sennerby *et al.*, 2008; Sennerby, 2008). In addition, these characteristics also allow for the combination with PDMS (Polydimethylsiloxane) microstamping via soft lithography; a technique extensively used in the last decade to produce ordered (isotropic or anisotropic) or randomized features, to engineer surfaces (Xia and Whitesides, 1998; Mata, Kim *et al.*, 2009; Kim, Boehm *et al.*, 2010) that can modulate cell behavior.

Such surfaces have the potential to induce positive cellular responses in terms of morphology, adhesion, migration, proliferation, and differentiation (Singhvi, Stephanopoulos *et al.*, 1994; von Recum and van Kooten, 1995; Chen, Mrksich *et al.*, 1997; Brunette, 1999; Flemming, Murphy *et al.*, 1999; Leonhardt, Renvert *et al.*, 1999; Turner, Dowell *et al.*, 2000; Walboomers and Jansen, 2001; Wilkinson, Riehle *et al.*, 2002; Brett, Harle *et al.*, 2004; Klinge, Hultin *et al.*, 2005; Kim, Shin *et al.*, 2008; Bettinger, Langer *et al.*, 2009; Pelaez-Vargas, Ferrell *et al.*, 2009a; Pelaez-Vargas, Ferrell *et al.*, 2009b; Pelaez-Vargas, Gallego-Perez *et al.*, 2010).

The present work describes the preparation of ordered micropatterned silica thin films on 3Y-TZP, intended to enhance guided tissue regeneration on ceramic dental implants. In order to understand how such a combination would perform, materials characterization and in vitro biological behavior in terms of early adhesion and proliferation of osteoblast-like cells were carried out.

## 2. MATERIALS AND METHODS

### a. Substrate processing and characterization

A synthetic 3 mol% $Y_2O_3$-$ZrO_2$ (3Y-TZP) powder having a nearly uniform submicron particle size was used (TZ-3YS-E, Tosoh, Tokyo, Japan). Chemical analysis of the powder was carried out by X-ray fluorescence spectroscopy (XRF, Magi X; Phillips, the Netherlands) and flame emission spectroscopy for alkaline analyses (FES, 2100; Perkin Elmer) to verify the values provided by the supplier. The specific surface area of the powder was determined by the chromatographic method (Monosorb Surface Area Analyzer MS-13, Quantachrome Corporation, UK) using a BET isotherm. Particle size distribution was measured using a laser scattering size analyzer (Master Sizer S; Malvern Instruments, UK). The particle size measurements were done on slurry samples. Dolapix CE-64 (Zschimmer & Schwarz GmbH & Co KG, Germany) was used as dispersant. Dispersion and de-agglomeration of particles were further ensured through ultrasonic treatment prior to measurement.

Cylindrical specimens were produced via uniaxial pressing, and their sintering behavior was evaluated using a high resolution dilatometer (Setsys 16/18, Setaram, France), at a heating rate of 5 °C /min from 20 °C to 1550 °C. Based on the thermal expansion/contraction data (dilatometer curve, Figure 24a), all discs were sintered at 1480 °C for 2 h. Contraction of all specimens

was calculated based in the comparison between the diameters of the "green" discs and of the full sintered discs. Maximum density was determined using the Archimedes water displacement method, while crystallography and full transformation from monoclinic to tetragonal phase was followed by XRD analysis (Siemens D5000, Germany). Visible Raman spectra (resolution= 4 $cm^{-1}$, acquisition time= 5s, 514 nm laser) were recorded using a confocal Raman microscope (LabRAM HR800 UV, Horiba Jobin-Yvon, Villeneuve d'Ascq, France), as a complement to confirm m→t phases transformation on powders, fully sintered discs, and re-heated discs (t→m phases transformation) (Zinelis, Thomas *et al.*, 2010).

The sintered samples were polished with a series of diamond pastes down to 1 μm. Microstructure observations were performed on polished and thermally etched surfaces via Field Emission Scanning Electron Microscopy (FE-SEM, Hitachi-S4700, Tokyo, Japan, fitted with energy X-ray dispersive spectroscopy, EDS, Noran System, Japan).

## b. Thin film production

Silica sol was produced via acid catalysis in a single step using tetraethylorthosilicate (TEOS, Aldrich, USA) and methyltriethoxysilane (MTES, Aldrich, USA) as precursors, following a previously reported method (Pelaez, Garcia *et al.*, 2004). The solution was aged for 24 h and applied via spin coating (3000 rpm for 45 s) on 3Y-TZP bare (3Y-TZP) substrates to produce flat silica coatings ($fSiO_2$).

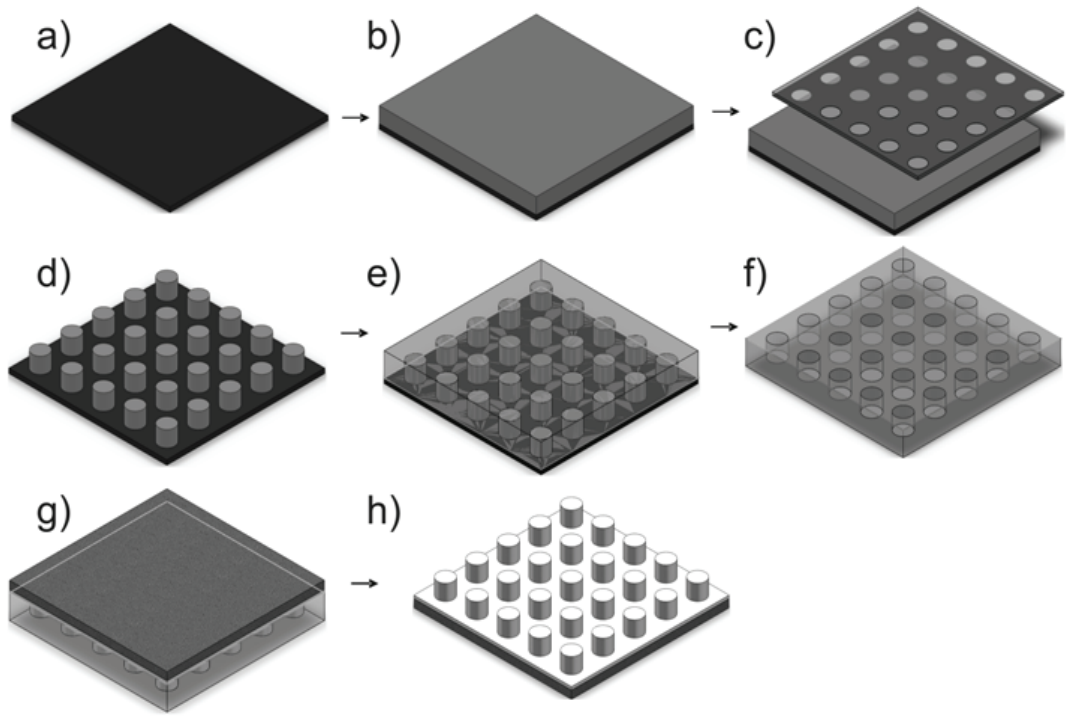



Figure 23. Schematic diagram of the microfabrication.

a) Silicon wafer, b) photoresist film is deposited, c) UV light exposure through the mask, d) patterned master, e) PDMS casting, f) PDMS negative mold, g) silica casting and drying and h) silica micropatterned thin film on 3Y-TZP.

Standard UV photolithography was used initially to create a silicon master with the pattern of interest. First, a chromium photomask was designed using L-Edit software (Tanner EDA, USA) and produced at a mail order mask-making foundry (Martin Photomask Service, USA). A negative tone SU-8 2005 (Microchem Corp, USA) photoresist was spin-coated on a silicon wafer at 3000 rpm to produce a ~5 μm thick film (Figure 23a,b). The photoresist was exposed to ultraviolet light through the photomask, and post-processed following previously established parameters (Figure 23c,d). Finally, the master was used to create PDMS (Silastic T2, Dow Corning, USA) negative molds (Figure 23e,f) with an ordered texture composed of pillars with 5 μm diameter, 5 μm height pillars, and spaced edge-edge by 5 μm.

3Y-TZP substrates were cleaned and dried. The sol was poured (40 µl) on the PDMS mold and the substrate was pressed against the sol (Figure 23g). After drying for 2 h, the mold was removed (Figure 23h). This technique is a modification of the pioneering work by Manzuri et al. (Marzolin, Smith *et al.*, 1998). Afterwards, the samples were evaluated under light microscopy, and then sintered at 500 °C for 60 min (Santucci, Di Nardo *et al.*, 1995) to produce micropatterned silica coating on 3Y-TZP substrates. The samples were characterized post-sintering via scanning electron microscopy (SEM).

### c. Cell Culture

MG63 cells (ATCC, USA) were cultured in α-minimal essential medium (α-MEM, Sigma, USA) containing 10% fetal bovine serum (ATCC, USA), penicillin-streptomycin (100 IU/ml and 10 mg/ml, respectively) (Sigma), gentamicin-amphotericin B (10 µg/ml and 0.25µg/ml, Invitrogen, USA), ascorbic acid (50 µg/ml, Sigma), and L-glutamine (2mM, Sigma). The cells were seeded at a density of $2x10^4$ cells/$cm^2$ on bare and silica coated (micropatterned and flat) zirconia and control group (TCP) samples. All cultures were incubated for 3 different time points (1, 4 and 7 days). Then, cells were washed twice with 37 ˚C PBS, fixed in 10% v/v neutral buffered formalin (Ted Pella, USA) for 15min, and prepared for SEM and epifluorescence microscopy (MIF).

### d. Morphology and Proliferation

For morphology evaluation via MIF (Eclipse TE2000-5, Nikon, Japan), the cells were washed and permeabilized with 0.1% v/v Triton X-100 for 30 min. F-actin filaments were stained using Alexafluor phalloidin (Invitrogen, USA) for 30 min. Nuclei were stained with a buffer of Propidium Iodide and RNase (BD Pharmigen, USA) at 4 °C for 15 min and washed with PBS.

For morphology evaluation via SEM, the cells were dehydrated in graded ethanol solutions and hexamethyldisilazane (HMDS, Ted Pella, USA) solutions (50% to 100%), respectively (Braet, De Zanger *et al.*, 1997). The samples were then gold-coated and visualized by SEM.

### e. Viability/Proliferation

Proliferation was evaluated by the alamar blue assay, a simple, non-reactive assay based in the oxidation-reduction of resuzarim, a non-fluorescent blue component that is reduced by living cells to a fluorescent pink component. Briefly, fresh medium with 10% alamar blue v/v (Sigma) was added, and incubated for 3 h. 100 µl of the medium was then transferred to a 96-well plate and a microplate reader (Infinite F-500, Tecan Trading AG, Switzerland) was used to quantify the fluorescence (at 535 nm excitation wavelength and 590 emission wavelength).

All cell culture experiments were carry out in triplicate (n=3) and results were expressed in relative fluorescence percentage of control group (TCP). Statistical analysis of the viability/metabolic activity was performed using one-way Anova/Tukey HSD post hoc test. Levels of $p<0.05$ were considered to be statistically significant.

## 3. RESULTS

The 3Y-TZP powder presents a specific surface of 6.2 $m^2/g$, with a mean grain size of 0.38 µm (d10= 0.19 µm, d50= 0.38 µm and d90= 1.17 µm). The crystallite size reported by the supplier was 36 nm. The result of the chemical analysis was (wt%(SD) oxide): 5.31(0.02) $Y_2O_3$; 0.244(0.003) $Al_2O_3$; 0.005(0.002) $SiO_2$; <0.002 $Fe_2O_3$; and 0.006(0.002) $N_2O$. These values agree with the ones reported by the supplier. Small traces of HfO2 were also detected.

Figure 24a shows the sintering curve of "green" compacted 3Y-TZP powders. The graph shows that the contraction started at around 1050 $^{o}$C (Figure 24a, single arrow), and it continued progressively up to 1500 $^{o}$C (Figure 24a, triple arrow), the highest contraction was reached at 1300 $^{o}$C (Figure 24a, double arrow). XRD analysis carried out on raw 3Y-TZP powders (Figure 24b) show two small peaks corresponding to the monoclinic phase (Figure 24b, arrows) and all the peaks of the major tetragonal phase. Optimum sintering temperature, to ensure high densification and full transformation of zirconia from monoclinic to tetragonal phase was established at 1480 $^{o}$C (Figure 24a, triple arrow), as confirmed by density data and XRD (Figure 24b). Raman spectra (Figure 24c) exhibited characteristic peaks at 146, 176, 188, 218, 260, 330, 377, 471 540, 557, 614 and 640 $cm^{-1}$ for raw powders, and peaks at 145, 260, 318, 400, 610 and 640 $cm^{-1}$ for fully sintered and re-heated discs, respectively (Figure 24c).

Geometric density of 3Y-TZP green-stage discs was $2.85 \pm 0.03$ g/$cm^3$, and the apparent density of sintered discs was $6.04 \pm 0.1$ g/$cm^3$, which is close to the X-ray theoretical density value of Y-TZP (6.07 g/$cm^3$) (Argyriou and Howard, 1995). A maximum contraction of 25% was seen during the thermal contraction analysis. This value is 3.5% higher than the diametral contraction observed on the discs (mean=21.5% and SD=0.67%).

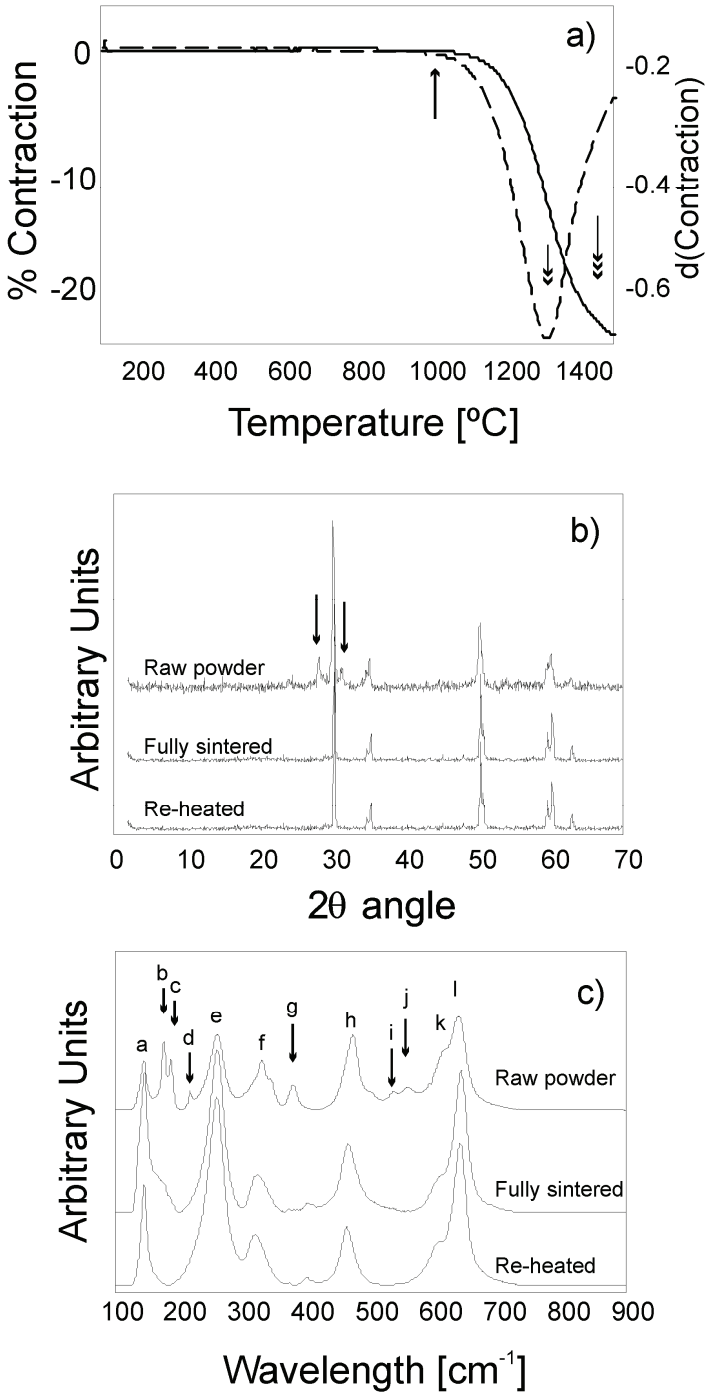



Figure 24. Thermal contraction, X ray Diffraction and Raman microanalysis.

a) Thermal contraction using a 5 ºC/min heating rate: absolute (dotted line) and differential contraction (continuous line). b) XRD diffraction and c) Raman spectra of raw commercial powders, and fully sintered or re-heated 3Y-TZPdiscs. Labels on the peaks correspond to defined wavelengths (a=146, b=176, c=188, d=218, e=260, f=330, g=377, h=471, i=540, j=557, k=614 and l=640 $cm^{-1}$)

SEM characterization (Figure 25c-d) shows that the tetragonal zirconia ceramic presents a dense and fine grained microstructure with a mean submicron grain size of 0.3±0.2 μm. No impurity concentrations (data not shown), at the level of resolution employed (FE-SEM fitted with energy X-ray

dispersive spectroscopy, EDS, Noran System, Japan), or singular large defects were found.

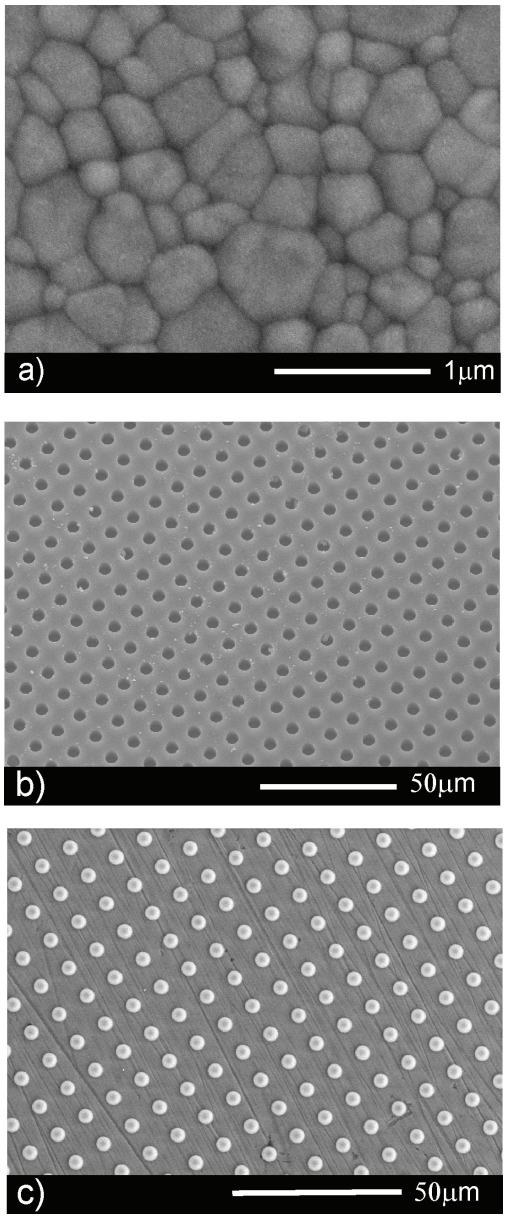



Figure 25. SEM images of Y-TZP substrate, PDMS mould and Silica coating on Y-TZP.

SEM images showing the 1 μm polished a) surface after thermal etching (1330 $^{o}$C for 30 min), the samples show a dense and $d_{50}$=0.3 (0.2 μm) fine grained microstructure of 3Y-TZP. b) PDMS mold and c) micropatterned silica coating with 5 μm diameter pillars on a 3Y-TZP substrate.

Figure 25b shows an SEM micrograph of a PDMS mold, with 5 μm diameter wells separated by 5 μm (edge-edge). Figure 25c shows a defect-free micropatterned coating. Polishing marks on the 3Y-TZP surface can be observed through the silica coating.

As the applied thin silica coatings needed to be sintered at 500 ºC, XRD analysis (Figure 25b) was conducted on a fully sintered disc, with an additional 500 ºC heat-treatment, to verify if there were no changes to the crystalline structure of the bulk material. This was confirmed by the similarity between Raman spectra of the fully sintered and re-heated specimens (Figure 24c).

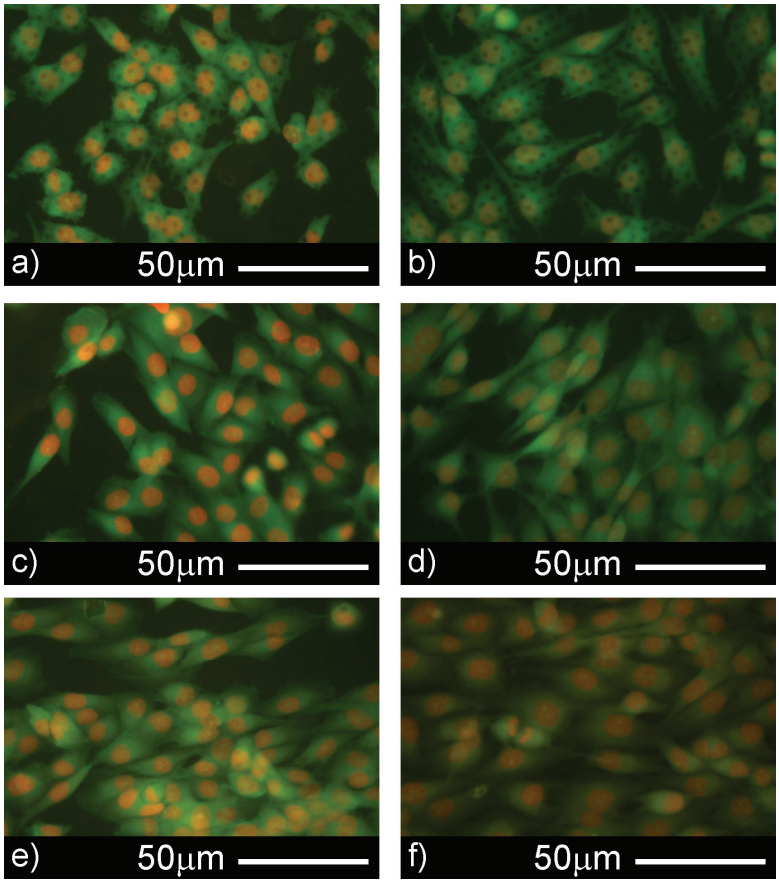



Figure 26. Early stages of adhesion and spreading of osteoblast-like MG63 on silica coatings. Micropatterned (a,b), flat silica coatings (c,d), bare 3Y-TZP (e,f) after 1d (a,c,e) and 4d (b,d,f) of culture. Scale bar = 50 μm.

Early stages of MG63 cell adhesion and propagation (1d and 4d) on flat and micropatterned silica surfaces, and on bare 3Y-TZP surfaces, are shown in Figure 26. Lamellopodia extensions can be observed on the micropatterned surface directed towards the micropillars. This effect is further enhanced at 4d of culture, when compared to what may be observed for flat silica or bare 3Y-TZP surfaces. However, there was more area surface coverage by the cells on both flat surfaces than on the micropatterned ones.

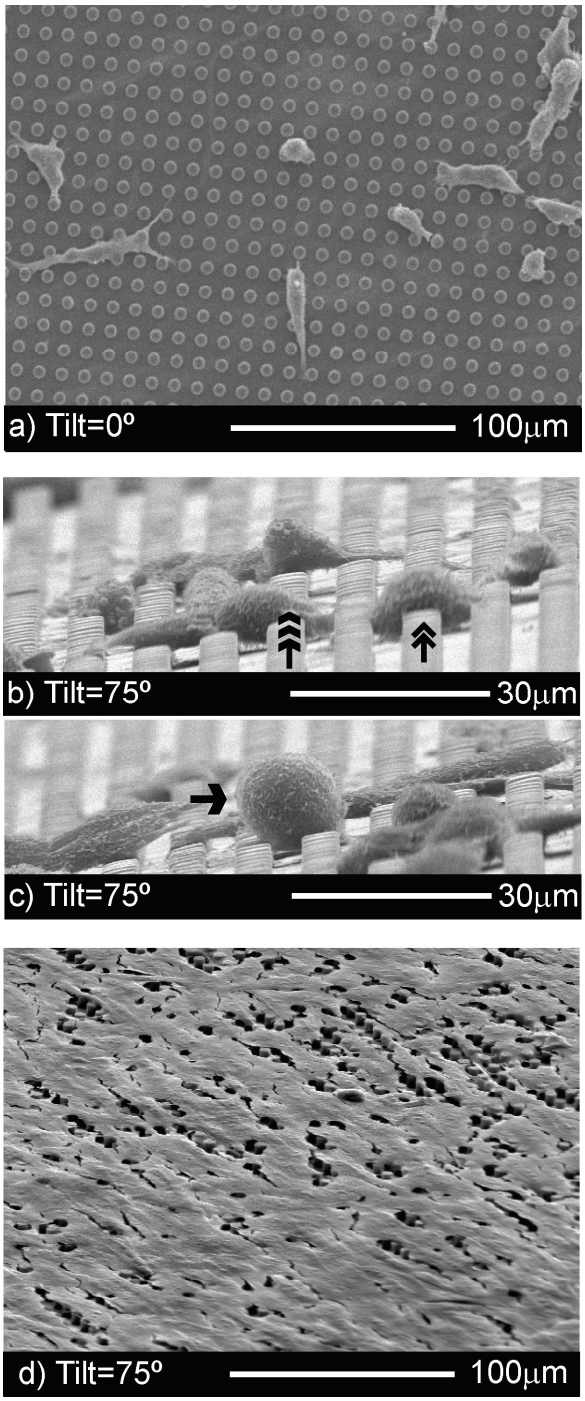



Figure 27. SEM images of adhesion and proliferation of osteoblast-like MG63 cells on micropatterned silica coatings. Cells with round shape (adhesion stage), elongated shape (spreading stage) at 1d (a-c). Monolayer of cells on the micropillar patterns was observed at 7 d (d,e).

Further characterization of the cells on the micropatterned surfaces via SEM (Figure 27a) revealed that during initial stages (t= 1d), some cells appear to exhibit a rounded morphology upon initial contact with the surface (Figure

27b-c single arrow), and an approximate aspect ratio of 2:1 between the cell and micropillars, which decreases to 1:1 for the cells already showing signs of initial spreading (double arrow) and subsequent alignment on the top of the pillars (triple arrow). Figure 27d shows low and high magnification images at 7-day cultures, where a dense cell monolayer growing on the top of the micropillars can be observed.

The alamar blue assay was selected to test the viability/metabolic activity of osteoblast-like MG63 cells on different surfaces. The one-way Anova test was used to evaluate differences in the percentage of viability/metabolic activity of MG63 cells on three different surfaces (using TCP as a control surface). This analysis showed a statistically significant difference between the surfaces ($F(6, 26)= 8.84$, $p<.001$) at all tested time points. Tukey post-hoc comparisons indicated that cells on micropatterned silica coatings (mpSiO2: M= 95.60%, SD=3.22%, p=.003) had a statistically significant higher metabolic activity than those on flat silica coatings (fSiO2: M= 89.27%, SD=1.91%) and bare Y-TZP (3Y-TZP: M= 92.50%, SD=2.67%) surfaces at 1d. No statistically significant differences were found at 4d (mpSiO2: M= 79.03%, SD= 1.77%; fSiO2: M= 73.82%, SD=5.43% and 3Y-TZP: M= 79.96%, SD= 6.27%). Finally, at 7d cells on micropatterned silica coatings showed a significantly lower activity (M= 61.90%, SD= 7.19%, p=.001) than cells on flat silica (M= 84.62%, SD=1.25%) and on bare Y-TZP (M= 81.85%, SD= 5.97%) surfaces.

## 4. DISCUSION

This study was proposed with the aim to microfabricate pillar patterned silica thin films on zirconia, using a strategy that combines soft lithography and sol-gel technologies. Based on several recent reviews on dental implants (Albrektsson and Lekholm, 1989; Albrektsson and Sennerby,

1991; Albrektsson, Sennerby *et al.*, 2008; Kohal, Att *et al.*, 2008; Sennerby, 2008), it is evident that titanium is the gold standard in clinical applications and many efforts have been proposed to improve its biological behavior through surface modification. It is also evident that there is a growing interest in zirconia implants but ceramics implants are in the initial stages of development and further research is required to produce an effective and competitive candidate to become a clinical alternative to titanium.

The $Y_2O_3$ stabilized tetragonal $ZrO_2$ polycrystalline ceramic (1480 $^oC$ by 2h), used in the present work, consists of homogeneous and fine equiaxed grains of tetragonal $ZrO_2$ (d50 = 0.3±0.2 μm) sintered to ~99% of the theoretical density. The small amounts of stabilizing Y2O3 (3 mol%) maintain the metastable tetragonal phase following sintering. This additive is uniformly distributed, forming a $ZrO_2$ solid solution as confirmed by FE-SEM/EDS. The homogeneous monophasic microstructure, as well as the lack of segregation of non equilibrium phases, indicates that the microstructure corresponded to chemical and microstructural equilibrium. The low intensity peaks in the Raman spectra of raw powders might be interpreted as monoclinic phase (176, 187, $cm^{-1}$), while fully sintered and re-heated discs (Figure 24b-c) exhibit the well defined high intensity peaks (146, 260, 325, 473, 614 and 641 $cm^{-1}$) corresponding to tetragonal phase (Palin, Fleming *et al.*, 2001; Ghosh, Suri *et al.*, 2006; Lughi and Clarke, 2007; Zinelis, Thomas *et al.*, 2010). According to the literature, this material could present high bending strength ($\sigma_f$ = 1000 - 1200 MPa) and toughness (KIC=6-9 MPa $m^{1/2}$), depending on the microstructure and critical flaw size. The reported values for hardness (Singhvi, Stephanopoulos *et al.*) and Young Modulus are around 13 and 210 GPa, respectively (Chevalier, 2006; Denry and Kelly, 2008).

After re-heating for one hour at 500 $^oC$, which is close to the eutectoid temperature (located at 4.5 mol% $Y_2O_3$ and 490$^oC$) for the $ZrO_2$-$Y_2O_3$ binary

system (Pascual and Duran, 1983), the eutectoid reaction (tetragonal zirconia solid solution = monoclinic + cubic zirconia solid solutions) was not detected. Therefore, de-stabilization of the tetragonal zirconia solid solution, in the bulk of the structure, did not occur. The retention of the tetragonal zirconia is explained by the slowness of the solid state reactions at such low temperatures. Visible Raman spectroscopy, a technique used to characterize both bulk and surface properties, confirmed that there were no differences in the crystalline structure between sintered and re-heated (500 °C for 1h) specimens. For further studies, UV Raman spectroscopy could be explored, as it is more sensitive for surface analysis (Palin, Fleming *et al.*, 2001; Ghosh, Suri *et al.*, 2006; Lughi and Clarke, 2007).

Surface modification on zirconia presents a technological challenge with few alternatives reported until now. In general, subtractive processes produce changes in the surface roughness. This type of surface modification is well studied on titanium, where an increased roughness has been reported to improve in vitro osteoblast adhesion/proliferation (Brett, Harle *et al.*, 2004), however, it also facilitates in vitro bacterial adhesion (Leonhardt, Renvert *et al.*, 1999; Klinge, Hultin *et al.*, 2005). From an *in vivo* perspective, strong adhesion of peri-implant soft tissues helps to prevent bacterial colonization and subsequent chronic inflammation, which increases the risk of developing peri-implantitis.

Zirconia surfaces modifications using additive techniques may increase bioactivity. Kim et al. (Kim, Kong *et al.*, 2004) reported the production of homogeneous fluoro-hydroxyapatite coatings with ~1 μm thickness on zirconia using a sol-gel approach. Their SEM images apparently showed a uniform interface without the presence of defects, intuitively indicating the occurrence of a chemical and mechanical compatibility between coating and substrate. In the present work, homogeneous crack-free flat silica thin films

were successfully obtained using spin coating. These films were optimized in a previous study using fractional factorial statistical experimental design, where a thickness dependence on the rotational speed, acceleration and aging of the sol was found (data not published).

Sollazo et al. (Sollazzo, Palmieri *et al.*, 2008) reported an interesting technique to introduce high roughness coatings on titanium or zirconia. In their work, zirconium dioxide was produced by colloidal precipitation, and deposited on the substrates by dip coating, followed by heat treatment at 700 $^{o}$C for 60 min. The biological behavior was evaluated both in vitro and *in vivo*. The animal study showed that the coating promoted increased bone integration (Sollazzo, Pezzetti *et al.*, 2008). The in vitro studies with MG63 cells and microarray technology showed promising results as well. However, even though their coatings showed appropriate thickness (~1 μm) for dental implants, it is also evident, based on their SEM micrographs, that the coatings presented adhesion problems and discontinuities (Sollazzo, Palmieri *et al.*, 2008). In the present work, microfabricated and flat silica thin films exhibited stable interfacial adhesion to the 3Y-TZP substrate.

Calcium phosphate glass-hydroxyapatite (HA) composites have also been studied as coatings on zirconia. Different mixture ratios were evaluated in terms of heat-treatment temperature, morphology, adhesion strength, solubility and cell responses. Their SEM images showed a highly dense and homogeneous continuous coating on zirconia substrates (Kim, Georgiou *et al.*, 2004). Proliferation and ALP activity of osteoblast-like cells on these surfaces were improved in comparison to bare zirconia. The cell proliferation results are agreement with our observations after 7 days of culture. This trend was also observed in a study about silica-containing HA nanospheres, where in addition, an optimum amount of $Si^{+}$ was found to increase the bioactivity without accelerating the coating dissolution (Thian, Huang *et al.*, 2006).

A host of biomedical applications using sol-gel technology have been published (Klein, 1985; McDonagh, Sheridan *et al.*, 1996; Hench, 1997; Garcia, Galliano *et al.*, 2003; Garcia, Cere *et al.*, 2004; Kim, Kong *et al.*, 2004; Pelaez, Garcia *et al.*, 2004). In the present work, sol-gel coatings were sintering at 500ºC for 1h, these conditions were selected in order to ensure effective removal of the ethyl and methyl groups from the alkoxides, producing a change from Si-O-$C_2H_5$ or Si-$CH_3$ to Si-O bonds, and formation of a complete $SiO_2$ lattice, as it was described by Santucci et al. (Santucci, Di Nardo *et al.*, 1995).

The introduction of microfabrication techniques into biomedical applications has allowed for the study of the effects of surface topography on cellular behavior, using polymers, silicon or metal thin films; materials that present mechanical disadvantages for structural applications in dentistry or orthopedics (Mata, Boehm *et al.*, 2002; Mata, Kim *et al.*, 2009; Kim, Boehm *et al.*, 2010). With current technology, the microfabrication of ceramics is associated with hard lithography techniques that in many cases are expensive, time consuming and have low yield. The combination of silica sol-gel chemistry and soft lithography was initially reported to produce optical waveguides and glass surfaces (Marzolin, Smith *et al.*, 1998), but to the best of the authors knowledge, there is a limited number of reports about the use of this approach for dental ceramics (Pelaez-Vargas, Ferrell *et al.*, 2009b)..

Considering the effects of the topography on cellular behavior. It is accepted that micro-engineered surfaces produce unique and reproducible cellular responses (Singhvi, Stephanopoulos *et al.*, 1994; von Recum and van Kooten, 1995); however, such behaviors are not universal for all types of cells and they are apparently associated with specific aspects such as origin, size and function of the specifically studied cells in each case. In this work, a

strategy to produce micropillared silica thin films on 3Y-TZP was presented. MG63 osteoblast-like cells showed a preference to adhere and grow on the top of micropillars at all time points. No apparent interactions were seen between the cellular processes and the sidewalls of the pillars, or the underlying flat surface. This preference was consistent with observations made by Turner et al. (Turner, Dowell *et al.*, 2000) where astrocyte-like cells were cultured on single-crystal silicon micropillars.

In the current work, viability/metabolic activity of MG63 osteoblast-like cells that was evaluated using an alamar blue assay was higher on the micropillars and flat silica coatings that on the 3Y-TZP surfaces. These results could be explained as being associated with the surface chemistry (Thian, Huang *et al.*, 2006), because of the high silicon content. No significant differences were seen between the patterned and flat coatings. Other studies have reported higher proliferation of human bone marrow cells on PDMS micropillars (Kim, Boehm *et al.*, 2010) than on flat surfaces. The differences between both findings could be due to differences in the stiffness of the substrate, material chemistry, and micropattern geometry. Studies on the effect of silica micropatterned geometry are currently being conducted by our research team, and will be addressed in future publications.

## 5. CONCLUSIONS

The current study described a technique that combined sol-gel and soft lithography to modify the surfaces of 3Y-TZP with micropatterned silica for the potential use in dental implants. This technique allowed the fabrication of micropillared silica surfaces with 5 μm diameter and 5 μm wide gaps between pillars, at low temperatures (500 $^{o}$C). In vitro studies showed that cells were able to adhere, elongate, migrate and proliferate on the coatings, preferably covering the top surfaces of the pillars, and mostly avoiding contact with the

bottom surface of the pillar side walls. This behavior indicated a cytocompatible pattern, alluding to their potential applications as coatings for use in dental implantology.

## 6. ACKNOWLEDGMENTS

This work was supported partially by the Portuguese Science and Technology Foundation (Scholarship FCT/SFRH/BD/36220/2007 and Grant FCT/PTDC/CTM/100120/2008 "Bonamidi"), CRUP - Acções integradas Luso-Espanholas E46/09, Acciones integradas Hispano-Portuguesas, MICINN: HP2008-0075. NSF Nanoscale Science and Engineering Center fellowship (NSF, Grant #EEC-0425626) and U.S. Air Force Office of Scientific Research MURI (Grant #F49620-03-1-0421). The support provided by Dr. C. Ribeiro (Raman microanalysis) is gratefully acknowledged.

## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

# Chapter 4. GENERAL CONCLUSIONS

This chapter contains the main statements, conclusions and some perspectives following the chapter organization in terms of basic concepts, ceramic substrates, flat and micropatterned silica thin films and biological behavior of different types of cells, as HBMSCs, Human Gingival Fibroblast, Pulp-derived MSCs and osteoblast-like cells, on the different developed surfaces.

The following statements related with aspects such as clinical need, and main techniques and materials used, that motivated the present work, may be produced:

1. Reconstruction of significant portions of the craniofacial skeleton and oral tissues are commonly required as consequence of caries, periodontitis, trauma, tumors and craniofacial anomalies.
2. Micro- and nanoscale technologies, derived mostly from the semiconductor technology, have been shown to offer unique capabilities to develop systems that can be used to control and promote cellular behavior in their specific microenvironments.
3. Microstamping is an unexplored technique adequate to modify surfaces of dental ceramic biomaterials with microstructured hard materials.
4. PDMS is an inexpensive and flexible material to produce features down to sub-micrometric range.
5. Dental ceramic materials is a subject still open to research in aspect such as esthetics, ceramic processing, biological activity (increasing its interaction with cells or reducing bacterial adherence) and surfaces modifications technologies.

From the work developed in **Ceramics substrates and silica coatings processing,** the following conclusions may be drawn:

1. The incremental ceramic mixing technique used to produce feldspathic porcelain specimens showed to retain lower water content in the green-state specimens, and to present higher biaxial flexural strength after sintered, decreased porosity and increased crystallinity, when compared with specimens produced by one-step mixing technique.
2. Zirconia framework cores produced by Procera System showed the highest roughness compared to those modified with a layer of pigment ceramic or with a multi-layer of aluminum Oxide Veenering Porcelain. This higher roughness did not affect the early adhesion of human gingival fibroblast.
3. Yttria stabilized zirconia used in the present work showed maximal contraction at 1300 $^{o}$C with complete transformation from monoclinic phase to tetragonal phase.
4. Thermal re-heating at 500 $^{o}$C that it is required to sinter the coating did not affect the Y-TZP substrate.
5. Isotropic, anisotropic and flat silica thin films between 200 µm to 500 nm were successfully developed.
6. Micropatterned hybrid silica thin films produced by sol-gel exhibited after sintering a contraction of 10.9% and small increases in the amplitude parameters of roughness.
7. Micropatterned hybrid silica thin films of different features shape and size, and intespacing were successfully produced on flat and on curved surfaces.
8. Synergy between sol-gel and microstamping to produce micropatterned silica coating was shown on different substrates

such as glass, feldspathic porcelain, In-Ceram alumina, Y-TZP, Stainless Steel and Titanium.

9. A successful technique to modify micropatterned silica coatings with bioactive particles of micro-aggregates of nanoHA or Portland Cement was implemented.
10. Flat silica coating, modified with bioactive particles, were more efficiently produced by dip coating than by stamping.
11. Multivariate experimental design allowed to develop an ad-hoc mathematical model to predict the thickness of silica coatings conditioned by solution aging, delay time and rotation speed. Statistical experimental design was an adequate tool to develop and optimize silica coatings with a reduced amount of experiments.

From the work developed in ***"in vitro"*** **cell studies,** the following conclusions may be drawn:

A general conclusion about the behavior of different cells evaluated in the present work, supports that silica thin film microtopography might lead to tissue guided regeneration, considering that these surfaces modulate cells response in terms of adhesion, spreading, cytoplasmic protrusions, proliferation, migration and motility.

More specific conclusions may be:

1. Isotropic silica coating did not exhibit a marked control on the alignment of human bone marrow stem cells at 4h and 24h. This cell type exhibited a significant elongation over micropillars.
2. Anisotropic silica coatings showed a clear polarization and alignment in the same direction of line-shaped patterns. After 7

days of culture, the cells increased the surface coverage but their proliferation presented a relative delay.

3. Hybrid silica thin films, both micropatterned and flat, showed to be biocompatible.
4. MG63 human osteoblast-like cells on pillar-shape micropatterned surface on 3Y-TZP showed a preferencial adhesion to the top of the pillars. At initial hours of culture they exhibited elongated plasmatic projections. After 7 days of culture, they fully covered the surface. This behavior may be comparable to that of the same cells on patterned coating modified with nanoHA.
5. SaOs-2 human osteoblast-like cells showed a marked alignment before 7 days of culture while metabolic activity and ALP activity increased at 21 days.
6. Human gingival fibroblast cultured on line-shaped thin films exhibited similar alignment, to that shown by other types of cells.
7. Pulp-derived stem cells showed directional adhesion and spreading on micropatterned silica coatings modified with nanoHA.
8. Pulp-derived stem cells showed a three fold higher motility and directionality on micropatterned surfaces when compared to flat surfaces. This result still requires further confirmation using other cell types from the oral cavity.

# APPENDIX

This section has different information related with the full research activity in the last six years including information in a width grade of consolidation (from preliminary to more mature work). The main richness in each information consolidated in this section, help us to discuss new ideas with researchers from different countries. Partially, some of these ideas have been work in this section. I think that they might be the best answer about the needs of future work.

# APPENDIX A. DENTAL CERAMICS SUBSTRATES

**Overview**

This appendix section is divided in five abstracts presented in the Annual Meeting of International Association for Dental Research in New Orleans (USA, 2007), Toronto (Canada, 2008) and Barcelona (Spain, 2010) and a manuscript rejected in Dental Materials and Journal of Dentistry.

- **Abstracts**

***In the first abstract***, entitled *"Microwave pre-drying effects on Feldspathic Porcelain - Biaxial Flexural Analysis"*. The purpose of this study was to compare the resistance to flexion of a feldesphatic ceramic submitted to pre-drying via microwaves previous to the sintering with respect to a control group. This abstract is referenced on Journal of Dental Research.

***In the second abstract***, entitled *"Aging of barbotine used In-Ceram alumina system during storage"*. The purpose of this study was to compare the structural reliability and microstructure of discs obtained by two different ways of storing barbotine using aluminium oxide infiltrated with glass. This abstract is referenced on Journal of Dental Research.

***In the third abstract***, entitled *"Structural effects of mixture techniques during the ceramic paste preparation"*. The purpose of this study was to study flexural strength and microstructure of feldspathic porcelain discs produced using two different preparation methods as a measure of their structural reliability. This abstract is referenced on Journal of Dental Research.

***In the fourth abstract***, entitled *"Microhardness Obtained with Two Ceramic Paste Preparation Methods"*. The purpose of this study was was to

compare micro-hardness of ceramic discs produced using two different preparation methods as a measure of their surface micro-defects. This abstract is referenced on Journal of Dental Research.

***In the fiveth abstract,*** entitled *"Zirconia microtextured surfaces using soft lithography"*. The purpose of this study was to develop a molding technique based in soft lithography to produce micropatterned zirconia surfaces. This abstract is referenced on Journal of Dental Research.

- **Papers**

***Ninth manuscript,*** entitled *"Microwave pre-drying in dental porcelain - Structural Reliability",* was developed early in the PhD formation. The aim of this work was to compare the structural reliability of a feldspathic ceramic ("selected as a model dental material") when pre-dried by microwaves before sintering, with respect to control group.

## Abstract 1. MICROWAVE PRE-DRYING EFFECTS ON FELDSPATHIC PORCELAIN - BIAXIAL FLEXURAL ANALYSIS

**PELÁEZ-VARGAS A**, CUARTAS JC, OSORIO L, MONTEIRO FJ. Microwave pre-drying effects on Feldspathic Porcelain - Biaxial Flexural Analysis. JOURNAL OF DENTAL RESEARCH. 2008;87(Spec Iss B):2322. (**www.dentalresearch.org**).

*2322 MICROWAVE PRE-DRYING EFFECTS ON FELDSPATHIC PORCELAIN - Biaxial flexural analysis*

http://iadr.confex.com/iadr/2008Toronto/techprogram/abstract_103606.htm

**PELÁEZ-VARGAS** A [1], J.C. CUARTAS [2], L. OSORIO [2], and MONTEIRO FJ [3].

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The structural reliability is one of the main objectives in the systematic fabrication of the dental ceramic products. The application of electromagnetic energy of the microwave type in the process of pre-drying of dental ceramics presents an attractive option to explore as a strategy of improving upon the mechanical properties of final pieces, and at the same time for diminishing the time of processing and for saving of energy.

Objectives: to compare the resistance to flexion of a feldesphatic ceramic submitted to pre-drying via microwaves previous to the sintering with respect to a control group.

Materials and methods: 75 feldspathic porcelain discs were prepared and divided themselves in three identical groups that correspond to the control group with no pre-drying via microwaves, a group pre-drying via microwaves in a single cycle of 10s, and a group pre-drying via microwaves in tree cycles of 10s with intervals of 1 min among themselves. All pre-

drying via microwaves were done using a conventional microwave oven. Discs were polished and evaluated with Biaxial flexural test (Figure 28).

Results: The results of asymmetrical analysis using Weibull model do not present differences with respect to the characteristic effort found: 59.47 Mpa, 56.07Mpa and 57.09 Mpa for groups I, II and III respectively.

The highest of Weibull module found I the group pre-treatment with microwaves during 30s (6.51) presents a more uniform group of ceramic discs and with more structural reliability.

Conclusions: To suggest a standardized protocol of ceramic processing that allows the obtainment of more similar pieces, and with less probability of failing because of microflaws.

Acknowledgment:
Program-Alban/E05D050652CO, FCT/SFRH/BD/36220/2007 and FCT/ POCI/ SAU-BMA/56061/2004.

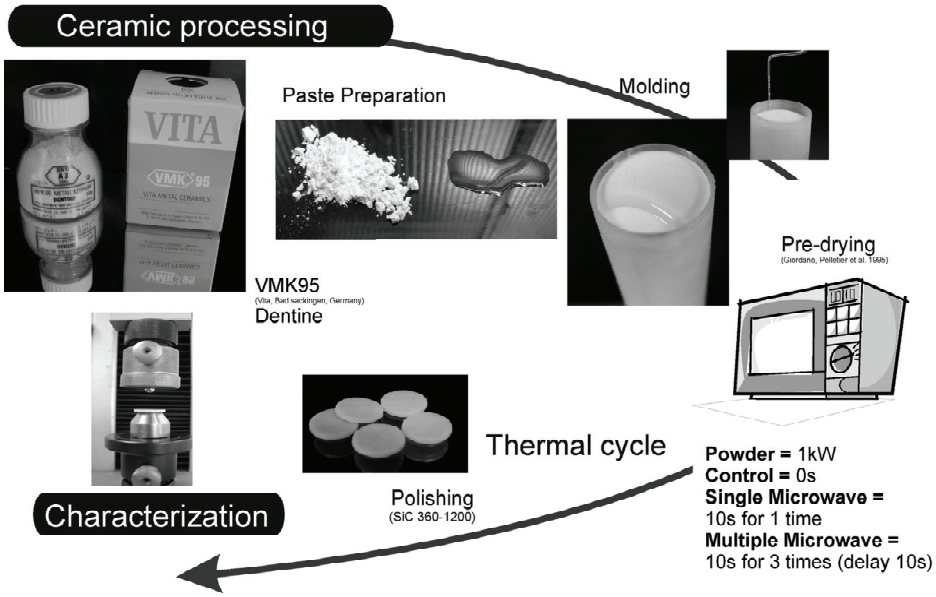



Figure 28. Diagram of ceramic processing and characterization of feldspathic discs evaluated.

## Abstract 2. AGING OF BARBOTINE USED IN-CERAM ALUMINA SYSTEM DURING STORAGE

**PELÁEZ-VARGAS A**, VILLEGAS A, BORJA M, RESTREPO LF, MONTEIRO FJ. Aging of barbotine used In-Ceram alumina system during storage. JOURNAL OF DENTAL RESEARCH. 2007;86(Spec Iss A):0880. (**www.dentalresearch.org**).

*0880 AGING OF BARBOTINE USED IN-CERAM ALUMINA SYSTEM DURING STORAGE*

http://iadr.confex.com/iadr/2007orleans/techprogram/abstract_88702.htm

**PELÁEZ-VARGAS A** [1], VILLEGAS A [2], BORJA M [2], **RESTREPO LF** [2], and MONTEIRO FJ [3].

[1] INEB - Instituto de Engenharia Biomédica, Laboratorio e Biomateriais, Porto, Portugal, Portugal.
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Introduction: All-ceramic systems are generally considered expensive for most dental laboratories in developping countries. Barbotine of In-Ceram Alumina System could be stored at -20°C with the purpose of minimizing its production costs. However, limited information exists on the possible effects that this practice has on the structural behavior of dental restorations.

Objectives: The purpose of this study was to compare the structural reliability and microstructure of discs obtained by two different ways of storing barbotine using aluminium oxide infiltrated with glass.

Methods: 50 alumina discs infiltrated with glass were produced and divided in two groups according to storage time of barbotine (0 and 30 days). The discs were polished and evaluated by biaxial flexural strength tests. Statistical studies included Weibull distribution and extreme values analysis. SEM and XRD were used to identify the crystalline phases of both groups. Chemical analysis was conducted by EDS and FTIR spectroscopy to detect differences between both groups.

Results: The characteristic strength obtained for the 0 days group was 20% higher than that of 30 days group. In the analysis of Weibull module no

relevant differences were noticed between both groups. Differences in glass infiltration were found by SEM (Figure 29), while no differences were found by EDS and FTIR.

Conclusions: It has been shown that, after 30 days storage at -20°C, barbotine showed significantly lower mechanical strength with respect to the same material prior to storage, inducing reduced mechanical strength effects on glass infiltrated alumina discs.

Acknowledgement: The work was carried out under a PhD grant from Alban Program. European Union (Scholarship E05D050652CO), which is acknowledged

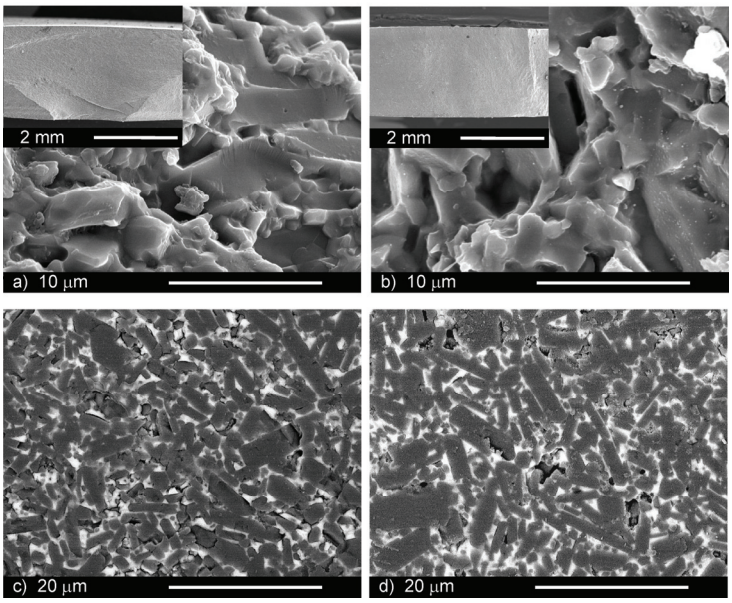



Figure 29. SEM Images of In-Ceram Alumina discs produced under diferents aging times. Zero days (a,c) and 30 days (b, d). Polished surfaces (c, d)

## Abstract 3. STRUCTURAL EFFECTS OF MIXTURE TECHNIQUES DURING THE CERAMIC PASTE PREPARATION

**PELÁEZ-VARGAS A**, DUSSÁN J, RESTREPO LF, MONTEIRO FJ. Structural effects of mixture techniques during the ceramic paste preparation. JOURNAL OF DENTAL RESEARCH. 2007;86(Spec Iss A):0380. (**www.dentalresearch.org**).

***0380 STRUCTURAL EFFECTS OF MIXTURE TECHNIQUES DURING THE CERAMIC PASTE PREPARATION***

http://iadr.confex.com/iadr/2007orleans/techprogram/abstract_88706.htm

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Introduction: Ceramic paste preparation is a fundamental phase in building up dental restorations. Usually the dental laboratory techniques include either an incremental or a "one step" mixture of powder and liquid, but the industry uses the second method with the purpose of obtaining minor micro-defects in the final material.

Objective: The aim of this work was to study flexural strength and microstructure of feldspathic porcelain discs produced using two different preparation methods as a measure of their structural reliability.

Methods: 100 Vita-Omega 900 porcelain discs were produced and divided according to ceramic paste preparation method, in a powder-liquid mixture group and "one-step" mixture group. Specimens were fractured in bold on ring test to measure their biaxial strength. (Figure 30). Samples were observed in SEM and XRD was used to identify the crystalline phases of both groups. Chemical and phase analysis was conducted by EDS, XRD and FTIR spectroscopy to detect differences between the two groups. Statistical analysis include Weibull distribution and extreme values analysis

Results: The mean average values were (74.61± 16.35 MPa, m= 6.74) and

(69.72 ± 12.71 MPa, m= 4.949) for powder-liquid and one-step mixture group respectively. No significant statistical differences were found between both groups. The percentiles evaluation for biaxial flexural strength has shown differences between both groups, this is more evident for lower values of strength. XRD comparison showed that more amorphous phase is present in the one-step group. SEM, EDS and FTIR evaluations did not show significant differences.

Conclusions: The one-step mixture technique showed lower structural reliability meaning that there is an increase in the heterogeneity of microdefects that may be associated with increasing amounts of amorphous phase being present and increased porosity.

Acknowledgement: The work was carried out under a PhD grant from Alban Program. European Union (Scholarship E05D050652CO), which is acknowledged.

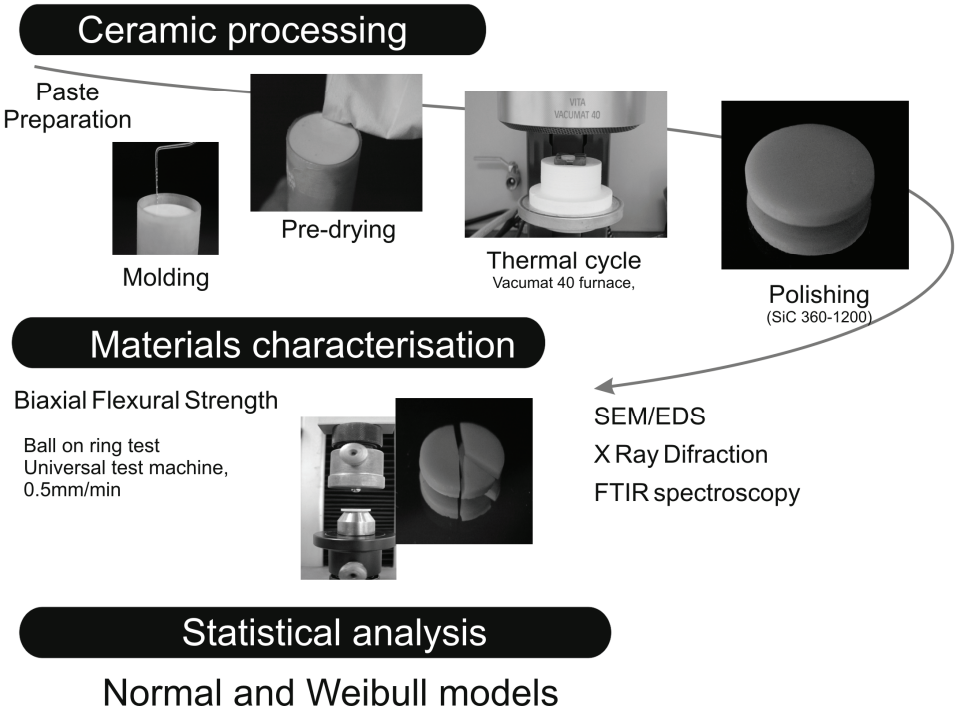



Figure 30. Ceramic Processing and characterization of discs produced by two different strategies of ceramic paste preparation.

## Abstract 4. MICROHARDNESS OBTAINED WITH TWO CERAMIC PASTE PREPARATION METHODS

**PELÁEZ-VARGAS A**, SALDARRIAGA J, ARANGO S, RESTREPO LF and MONTEIRO FJ. Microhardness Obtained with Two Ceramic Paste Preparation Methods. JOURNAL OF DENTAL RESEARCH. 2007;86(Spec Iss B):0294. (**www.dentalresearch.org**).

### *0294 MICROHARDNESS OBTAINED WITH TWO CERAMIC PASTE PREPARATION METHODS*

http://iadr.confex.com/iadr/israel07/techprogramforcd/A95753.htm

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Introduction: Dental ceramics show low structural reliability in both laboratory and clinical conditions due to ceramic processing. The ceramic paste preparation is a fundamental phase in building up the restorations. Usually the dental laboratory techniques include either an incremental or a "one step" mixture of powder and liquid, but the industry uses the second method with the purpose of obtaining minor micro-defects in the final volume.

Objectives: The aim of this study was to compare micro-hardness of ceramic discs produced using two different preparation methods as a measure of their surface micro-defects.

Methods: 100 Vita-Omega 900 porcelain discs were produced and were divided according to ceramic paste preparation method in an incremental mixture group and one-step mixture group (Figure 31). The discs were polished and tested according to Vickers hardness under a 4.905 N load during 15 seconds.

Results: Better resistance to indentation was found on the discs obtained by the incremental mixture method compared to one-step mixture method ($p<0.05$). But Weibull analysis shows that the one-step method allows for

less variability of surface micro-defects in the final samples.

Conclusions: Surface micro-defects inherent to processing are responsible for catastrophic failures of ceramic materials. It was found that better structural reliability of ceramic restorations might be achieved by using the one-step mixture preparation method.

Acknowledgement: Program Alban, the European Union Program of High Level Scholarships for Latin América, scholarship No.E05D050652CO and CIDI, Universidad Pontificia Bolivariana, Convocatoria 2005.

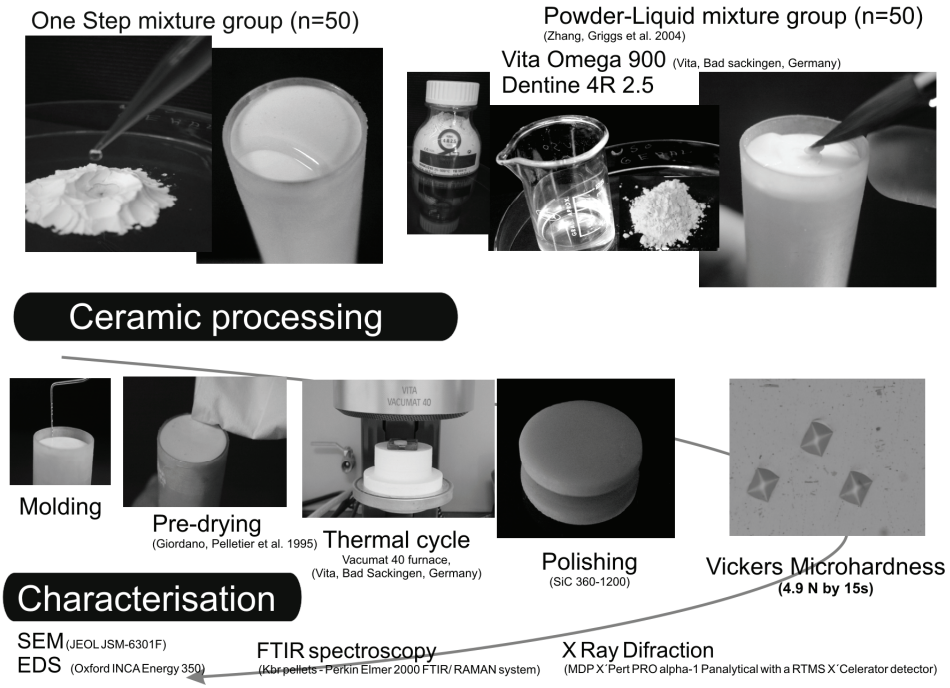



Figure 31. Ceramic processing of feldspathic discs and characterization by microindentation, FTIR spectroscopy, X-Ray diffraction and SEM/EDS.

# Manuscript 9. MICROWAVE PRE-DRYING IN DENTAL PORCELAIN - STRUCTURAL RELIABILITY

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*Rejected in Dental Materials and Journal of Dentistry. Some changes are necessaries to submit for a new journal.*

## ABSTRACT

Context. Structural reliability is one of the main objectives in the systematic fabrication of the dental ceramic products. Microwaves electromagnetic energy used to pre-dry dental ceramics is an attractive process to improve mechanical properties of dental ceramic final specimens. Objective: The aim of this work was to compare the structural reliability of a feldspathic ceramic ("selected as a model dental material") when pre-dried by microwaves before sintering, with respect to control group. Materials and methods: 75 feldspathic porcelain discs were prepared and divided in three identical groups corresponding to the control group with no pre-drying via microwaves, a group with pre-drying via microwaves in a single cycle of 10s, and a group with pre-drying via microwaves in tree cycles of 10s with intervals of 1 min between themselves. All pre-drying via microwaves was done using a conventional microwave oven. Discs were polished and evaluated with Biaxial flexural test and characterised using SEM/EDS and FTIR. Results: The results of asymmetrical analysis using Weibull model do

not present differences with respect to the characteristic strength between all groups. The highest values of Weibull module was obtained with multiple microwaves cycles. These values mean that multiple microwaves cycles group presents a more homogeneous distribution of flaws and higher structural reliability. Clinical relevance: To suggest a standardized protocol of ceramic processing using microwave pre
drying that allows the obtaintion of dental restorations with less and better dispersed flaws, leading to reduced catastrophic failure in service.

## 1. INTRODUCTION

Ceramics have been considered as the dental restorative materials with the best technology/clinical application ratio in the last decades due to their properties, as aesthetics, biocompatibility, wear resistance, (Anusavice, 1992) chemical and colour stabilities. However, their brittle behavior and catastrophic failure under complex stress under physiological and pathological loading during oral function are the main problems for their long term durability (Kelly, Nishimura *et al.*, 1996; Rosenblum and Schulman, 1997; Jung, Wuttiphan *et al.*, 1999).

During ceramic processing the ceramic paste preparation (Zhang, Griggs *et al.*, 2004) is followed by shape modeling, pre-drying, sintering and polishing. Pre-drying is a complex process poorly controlled in its first stage, at room temperature, because it is human know-how dependent. In the second stage, the conventional drying of green parts is completed in the early moments of the thermal cycle. A thermal gradient is established between surface and core, and consequently the water is initially displaced inward and later outward as vapour, this process produces a non-homogeneous distribution of flaws and lower structural reliability. The present study was

designed under the hypothesis that pre- drying by microwave frequency of green piece of dental porcelain might improve the mechanical properties of final pieces.

Microwave radiation has been used in ceramic application in different processes that include drying, slip casting and sol-gel processing, calcinations, sintering and joining of ceramics pieces (Metaxas and Binner, 1990). Their dental applications include sintering of Alumina, polymerization, drying, desinfection and production of controlled porosity of different materials (Baysan, Parker *et al.,* 1998; Yap, Yap *et al.,* 2003; Kutty, Bhaduri *et al.,* 2004; Klinge, Hultin *et al.,* 2005; Riakhovskii, Poiurovskaia *et al.,* 2006; Vasquez, Ozcan *et al.,* 2008).

In the microwave frequency range there are primarily two physical mechanics trough which energy can be transferred to a ceramic material which are the flow of conductive currents and re-orientation or dipolar loss (Metaxas and Binner, 1990). In the range of 1 to 10 GHz energy absorption is primarily due to the existence of permanent dipole molecules that tend to re-orientate under the influence of a microwave electric field. The origin of this heating effect lies in the inability to produce a permanent polarization, inducing an extremely rapid reversal of the electric field and vibration of the water molecules. Consequently, it is possible to produce an uniform thermal profile that is directed towards the surface and theoretically it produces a lower displacement of matter and a more homogeneous production of flaws (Brosnan, Messing GL *et al.,* 2003).

The aim of this work was to compare the structural reliability of a feldesphatic ceramic pre-dried by microwaves before sintering, with respect to a control group.

## 2. MATERIALS AND METHODS

In this study low fusing porcelain material (VMK95 Dentin, Vita, Bad Sackingen, Germany) was used to produce 75 discs, with 13mm diameter and 2mm height. The samples were divided in three groups of 25 discs each, that correspond to the control group with no pre-drying via microwaves, a group with pre-drying via microwaves in a single cycle of 10s, and a group with pre-drying via microwaves in three cycles of 10s with intervals of 1 min between themselves. All specimens were made from ceramic slurry, obtained by single addition of the liquid to the powder, using 1.82 g of powder per 0.66 ml of distilled water (Fleming, Shaini *et al.,* 2000; Zhang, Griggs *et al.,* 2004).

The ceramic paste was mixed at room temperature in a glass tile and then loaded onto a syringe with a ceramic brush, the ceramic was condensed using vibratory table and absorbent towel paper to remove the water excess (Giordano, Pelletier *et al.,* 1995). The plunge of the cut syringe was placed at 3mm from the edge using a periodontal probe, the discs surface was leveled against a flat surface to have a uniform thickness before removing the disc from the syringe (Zhang, Griggs *et al.,* 2004).

The green disc was removed from the syringe with a Number 15 scalpel and deposited on a refractory platform (Vita, Bad Sackingen, Germany). All microwave pre-drying cycles were made using a conventional microwave (Multimode applicator device, 1KW, 2450 MHz) before sintering according to the manufacturers recommendations.

The discs surfaces were polished with a series of abrasive papers (400,600 until 1200 grains), using a Stuers LaboPol-5 polisher (Zeng, Oden *et al.,* 1996). The discs thickness was measured with a digital caliper (Mitutoyo).

Biaxial flexure strength was determined by ball on ring test. Each disc was supported on a steel ring (9.86 mm diameter). Load was applied centrally through a spherical ball indenter (5 mm diameter) in an universal test machine with a cross-head speed of 0.5mm/min until failure (Test resource 656c, USA).

The maximum strength of the biaxial flexure test for each disc was calculated according to the equation:

$$\sigma_{max} = \frac{P}{h^2}\left[(1+\nu)\cdot(0.485\cdot l_n(\frac{a}{h}) + 0.52) + 0.48\right]$$

Where $\sigma_{max}$ is the maximum tensile strength measured in MPa, P the measured load at fracture in Newton, $h$ is the thickness of the disc measured in mm, $\nu$ is the Poisson's ratio - 0.25 for the porcelain (Fleming, Shaini *et al.*, 2000) and $a$ is the radius of the supporting ring in mm.

Weibull distribution was used to calculate probabilities of fracture for each group of samples as a function of the applied stress. The p-p plot of each group was obtained by Tukey's proportion estimation in order to verify if the Weibull model presented an adequate distribution of the experimental data. The model is considered as representative if the "Observed cumulative probability" and the "Expected cumulative probability", fit a straight line. (Figure 32)

The equation of the Weibull two-parameter distribution function used was:

$$P_f = 1 - \exp\left(-\int_s \left(\frac{\sigma}{\sigma_0}\right)^m dS\right)$$

Where $\sigma$ is stress; $\sigma_{max}$ is a scale parameter or characteristic strength (stress level at which 63.2 % of the specimens have failed); $S$ is the surface or volume under stress; and $m$ is a shape parameter, a dispersion parameter or Weibull modulus that is a constant which determines the slope of the

distribution. A high Weibull modulus is desirable for all materials since it indicates and increased homogeneity in the flaw population and a more predictable failure behavior.

The materials characterisation was done in terms of chemical composition and fractography analysis for the three studied groups.

The surfaces of discs and fractured cross-sections were analysed using Scanning Electron Microscopy (SEM) and EDS. Samples were sputter coated with gold using a JEOL JFC-100 fine coat ion sputter device. The discs were studied with a JEOL JSM-6301F microscope at an accelerating voltage of 15 kV.

Chemical characterisation was performed using Fourier transformed infrared (FTIR) spectroscopy. For this purpose, discs were evaluated using a PerkinElmer System 2000 spectrometer (Zhang, Griggs *et al.,* 2004) with an attenuated total reflectance (ATR) accessory (Golden Gate Single Reflection Diamond ATR, Specac, Smyrna, GA, USA). This method provided a qualitative analysis of chemical groups within 2 µm of the surfaces of monolithic specimens. The transmission range of the ATR accessory employed was 4000–400 $cm^{-1}$. The spectra were obtained at 4 $cm^{-1}$ resolution by averaging 100 scans per specimen group.

## 3. RESULTS

Figure 32 shows the good fitting of the experimental values obtained for the Biaxial Flexural Strength for the three groups, with respect to the Weibull Theoretical Model.

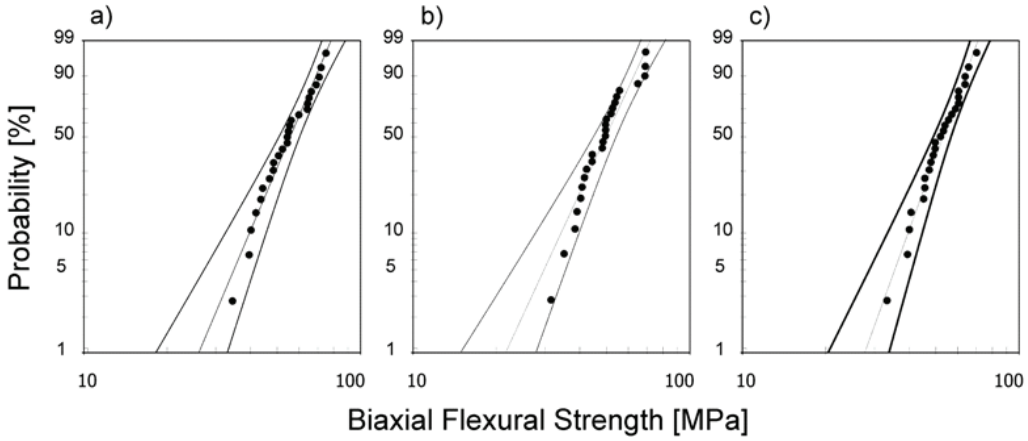



Figure 32. Weibull p-p plot for three groups evaluated.

a) Control, b) Single Pre-drying Cycle and c) Multiple Pre-drying Cycles. All experimental data present a good fitting with respect to the Weibull Theoretical Model.

**TABLE 7. WEIBULL ANALYSIS OF THREE DIFFERENT TIMES OF MICROWAVE PRE-DRYING**

| Pre-drying | n | Characteristic Strength [MPa] | Weibull module |
|---|---|---|---|
| Control [0s] | 25 | 59.47 | 5.57 |
| Microwave [10s] | 25 | 56.07 | 5.09 |
| Microwave [30s] | 25 | 57.09 | 6.51 |

Results of Weibull analysis are listed in Table 7. The Weibull module values found for all groups are low, but the higher value in multiple pre-drying microwave cycles group was found compared with controlled others groups indicating a slightly more uniform material with higher structural reliability.

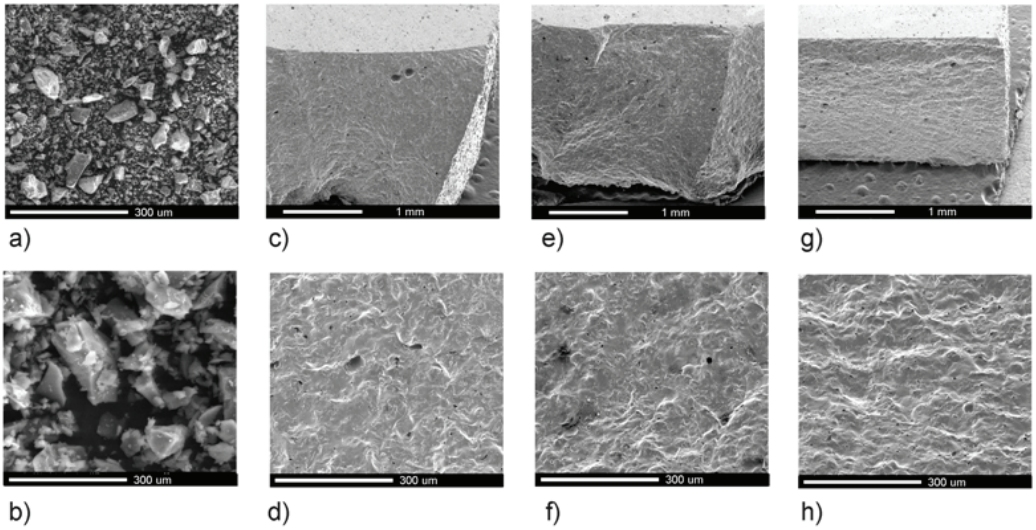



Figure 33. SEM images of VMK95.

(a,b) VMK95 powder, (c,d) fracture surfaces of control and (e,f) pre-dried specimens using a single cycle of microwaves (10s) and (g,h) using multiple cycles (10s x 3 times)

No significant differences in terms of fractography were found in the SEM analysis (Figure 33) between specimens deriving from three groups. However, the pores size distribution may be more homogeneous in the group of microwaves multiples cycles that in the others. No differences in the elemental analysis were found between all groups using EDS (data not shown).

Table 8 shows a comparison of the FTIR attenuated reflectance spectra for three different times of microwave pre-drying. All groups exhibit IR absorption bands with peaks at 446, 723, 995, 1448 $cm^{-1}$. All peaks were magnitude reduced in the following decrease order: Control group, Single microwave cycle and multiple microwave cycle.

**TABLE 8. FOURIER TRANSFORM INFRARED ATTENUATED REFLECTANCE SPECTRA OF SINTERED DENTAL PORCELAIN DISCS PRODUCED USING THREE DIFFERENT TIMES OF MICROWAVE PRE-DRYING**

| Group | Area [A.cm$^{-1}$] in the band position [cm$^{-1}$] | | | | Relative % |
|---|---|---|---|---|---|
| | Si-O-H | Si-O | Si-O-Si | Al-O | |
| | 1448 | 995 | 723 | 446 | [Si-O-Si/Total Si] |
| Control | 21 | 297 | 50 | 24 | 14 |
| Single Microwave Cycle | 22 | 296 | 53 | 30 | 14 |
| Multiple Microwave Cycles | 23 | 297 | 31 | 11 | 9 |

## 4. DISCUSSION

The ceramic processing includes several stages that are controlled by the manufacturing, however after production of high quality powder, with controlled particle size and complex chemical composition, other important steps are carried out by dental technicians and dentists, including the obtention of ceramic paste, molding, drying, sintering and polishing. These steps are essential to obtain a good structural reliability (Fleming, Shaini *et al.*, 2000).

During the modeling an empirical consistency of ceramic paste is a subjective characteristic depending on the operator's feeling, and results variability between different technicians is inevitable (Palin, Fleming *et al.*, 2001). However, optimized powder/liquid ratios have been reported for different ceramics (Fleming, Shelton *et al.*, 1999). It is accepted that liquids in optimized ratios occupy the spaces between ceramic particles, helping in achieving maximum packing by transport phenomena and by surface tension, lubricating particles during movement and leading to higher densities (Zeng, Oden *et al.*, 1996; Fleming, Shaini *et al.*, 2000). However, excess water present in the "green" piece must be avoided, because during the initial stages of

thermal cycle this is transformed into vapour, producing empty spaces that can lead to catastrophic failure by crack propagation.

Characteristic strength comparison does not show differences between the three groups. It is accepted that values lower that 10 for the Weibull module mean low structural reliability, but is should not be expected that dental feldspatic porcelain should present high values of Weibull module. However, differences found between microwave pre-drying procedures and control are relevant, considering that even small changes in the Weibull module associated to multiple microwave cycles pre-drying conditions, induce different flaw distribution and therefore better structural reliability.
Probably, the differences in the Weibull module between multiple microwave cycles group and control group is related with a low vapour flow and minor voids presence that produce a lower strength in green specimens during the first phase of drying (Gill, 1932).

Fleming et al (Fleming, Shaini *et al.*, 2000) showed that changes in porosity and bulk density, associated to small differences in the amount of water present, induce changes in the structural reliability.

Giordano et al (Giordano, Pelletier *et al.*, 1995) evaluated bar specimens under Four Point Bending using In-Ceram ceramic Slip Casting components in three groups non-fused alumina, fused glass, fused alumina that were compared with Dicor all-ceramics restorative systems and a Feldspathic porcelain (VMK68, Vita Zahnfabrik). Their results showed a lower mean value in bending ($69.74 \pm 5.47$ MPa), following the same trend of the results obtained in this work with the two evaluated methods of pre-drying and the control group. They obtained evidence by SEM analysis supporting that in the analysed feldespathic ceramic failure had started in defects produced during processing. In their report only 15 specimens by group were used and their

comparison was done in terms of Normal Distribution, these two aspects inhibit an exhaustive comparison to be established.

Our results were in agreement with those found for felsdespatic porcelain (Vita Alpha Dentin, Vita Zahnfabrik, Bad Sackingen, Germany) in terms of the mean strength 60.9 MPa with m module of 5.26. The structural reliability was not increased (Cattell, Clarke *et al.*, 1997).

The flexural strength values found in this study compared favourably with the work of other authors, taking into account variations in testing and specimen parameters. The transverse strength of Vitadur N dentine porcelain was within the flexural strength values of ~60MPa (Oilo, 1988) and 62MPa (Seghi, Daher *et al.*, 1990) quoted for feldspathic dentine porcelains.

Others authors showed that the presence of $H_2O$ in the structure of $K_2O$-$Al_2O_3$-$SiO_2$ causes them to crystallize much easier and more rapidly than the "dry" formulations. The presence of dissolved water (as hydroxyl) has been shown to induce crystallization at elevated temperatures (Ray and Day, 1996)..

The concentration of water in silicate glasses has also a decisive influence on viscous flow, generating differences in the viscosity data and in the predicted crystal growth rates (Burgner and Weinberg, 2001; Deubener, Muller *et al.*, 2003).

The IR absorption peak at 723 $cm^{-1}$ correspond with the vibration of (bridging) oxygen (Si-O-Si) bonds with the glass network. The IR absorption peak in the 446 $cm^{-1}$ corresponds to vibration of oxygen (O-Al-O) bond. The typical peak in 2926 corresponds to a (O-C-O) normally observed in this technique.

The broad peak from 760 to 995 $cm^{-1}$ results from the combined vibrations of non-bridging oxygen (Si-O-) associated with alkali and alkaline ions, as $Na^{+}$, $K^{+}$ and $Ca^{2+}$ in the glass network. This difference supports the interpretation that a thin glass layer was formed on the surface of specimens.

FT-IR was used to check this possibility in bulk specimens but no peak was detected to allow us to support the presence of molecular water in these specimens.

Following the results of this work it may be concluded that multiple microwave cycles increased the structural reliability of specimens produced using dental felsdespathic porcelain. These findings may show a new approach to the use of microwave in dental ceramics processing, with improvements in production time, costs, thermal uniformity, moisture control, but also in mechanical reliability

## 5. ACKNOWLEDGMENTS

The authors acknowledge the Support of the Eurodent, Bogotá, Colombia, Alban Program, the European Union Program of High Level Scholarships for Latin América, scholarship (No. E05D050652CO) and Portuguese Science and Technology Foundation (Scholarship number: FCT/SFRH/BD/36220/2007 and Contract number: FCT/POCI/SAU-BMA/56061/2004).

## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

## Abstract 5. ZIRCONIA MICROTEXTURED SURFACES USING SOFT LITHOGRAPHY

**PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, HIGUITA-CASTRO N, HANSFORD DJ, and MONTEIRO FJ. Zirconia microtextured surfaces using soft lithography. JOURNAL OF DENTAL RESEARCH. 2010;89(Spec Iss B):4004. (**www.dentalresearch.org**).

***4004 ZIRCONIA MICROTEXTURED SURFACES USING SOFT LITHOGRAPHY***

**http://iadr.confex.com/iadr/2010barce/webprogram/Paper140592.html**

**PELÁEZ-VARGAS A** [1], GALLEGO-PÉREZ D [2], HIGUITA-CASTRO N [2], HANSFORD DJ [2], and MONTEIRO FJ [3].

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All ceramic dental implants have been developed like an aesthetic alternative to metallic dental implants. However, the processing techniques commonly used to modify the surface of titanium implants are unable to modify surfaces of ceramics like yttria stabilized zirconia.
Microfabrication processing based in PDMS micromolding is an interesting alternative to produce engineered order topography to modulate tissue response.

Objectives: The purpose of this work was to develop a molding technique based in soft lithography to produce micropatterned zirconia surfaces.

Methods: Yttria stabilized zirconia (Y-TZP) commercial raw powders were used to produce slurries with different ratios of additives such as lubricants and aglomerants. Photolytography was used to produce a multilevel resolution mask with feature sizes starting from 500 nm up to 100 µm. PDMS negative molds were obtained. Soft pressing and centrifugation were used as methods for casting. Characterization was conducted using SEM/EDS.

Results: An optimized and castable ceramic slurry was successfully

produced, using low percentages of additives, as Polyvinyl Alcohol (PVA) and Polietilenglicol (PEG).

Y-TZP surfaces were obtained with, lines, cones and serpentine shapes, that were molded by soft lithography process. Optimized process was conditioned by shape factors thus being unable to reproduce pillar shape features due to mold extraction limitations (Figure 34).

Conclusions: Soft lithography enabled to produce microtextured Y-ZTP surfaces with features presenting a lateral resolution around 10 µm. A relevant restriction related with this technique was the occurrence of failures in "green" pillar shapes features during drying and de-molding process.

FCT grant FCT/SFRH/BD/36220/2007, Project Bonamidi PTDC/ CTM/100120/2008 and AFOSRMURI F49620-03-1-0421 are acknowledged.

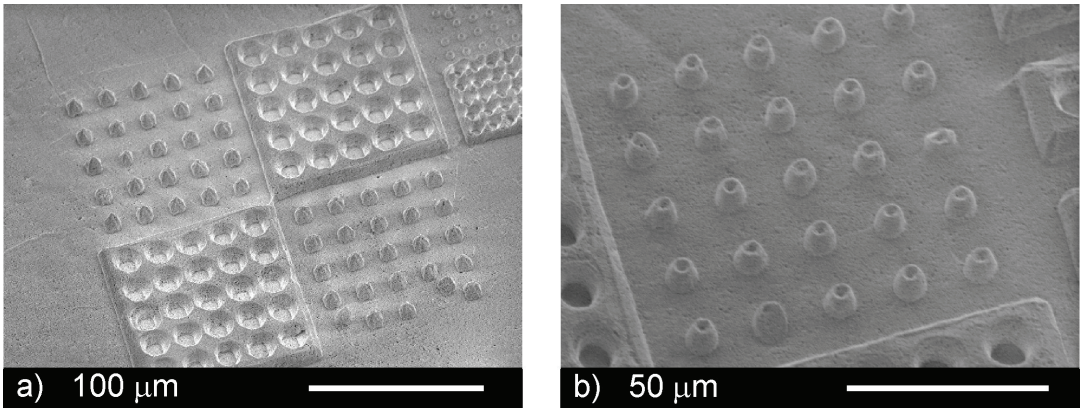



Figure 34. SEM images of Y-TZP microtextured surfaces from Resolution Mask

# APPENDIX B. SILICA THIN FILMS

**Overview**

This appendix section is divided in four abstracts presented in the Annual Meeting of International Association for Dental Research in Miami (USA, 2009) and Barcelona (Spain, 2010) and a manuscript rejected in Dental Materials and Journal of Dentistry.

- **Abstracts**

***In the sixth abstract***, entitled *"Microstamped Silica Films on Curved Zirconia Surfaces"*. The purpose of this work was to develop a molding technique based on soft lithography to produce anisotropic microtextured silica thin films on curved zirconia surfaces.. This abstract is referenced on Journal of Dental Research.

***In the seveth abstract***, entitled *"Portland Particulated Silica Coatings – Comparison Between Two Coating Techniques"*. The purpose of this work was to compare spin and dip coatings methods to produce silica thin films containing carbonated Portland cement particles for dental implants. This abstract is referenced on Journal of Dental Research.

***In the eighth abstract***, entitled *"Nanoparticulated HA/silica thin films on Zirconia surfaces"*. The purpose of this work was to develop silica thin films containing HA nanoparticles aggregates on zirconia surfaces. This abstract is referenced on Journal of Dental Research.

***In the ninth abstract***, entitled *"S. mutans and osteoblast adhesion on silica coatings"*. The purpose of this work was to evaluate adhesion of S. mutans and

Human Bone Marrow (HBM) cells cultured on silica thin films produced by sol-gel. This abstract is referenced on Journal of Dental Research.

***In the tenth abstract,*** entitled "*Producción de texturas por microestampación sobre cerámicas dentales*". The aim of this work was "*Producir texturas sobre superficies de biomateriales dentales utilizando microestampación y evaluar los efectos de la textura sobre comportamiento in vitro de fibroblastos gingivales y osteoblastos*". This abstract is referenced on the Abstract book of "*Sociedad Española de Cerámica y Vidrio*".

- **Papers**

***Tenth manuscript,*** entitled "*Bacterial adhesion and osteoblastic behavior on silica coatings optimized using Statistical Experimental Design*". This study concerns the thickness optimization of sol-gel coatings deposited by spin coating, using a statistical experimental design approach, as the use of this tool aims at reducing the amount of experiments without decreasing the quality of the information. The lowest thickness was selected as target of the optimization under the hypothesis that it could helps to reduce adherence failures.

## Abstract 6. MICROSTAMPED SILICA FILMS ON CURVED ZIRCONIA SURFACES

**PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, HIGUITA-CASTRO N, HANSFORD DJ, and MONTEIRO FJ Microstamped Silica Films on Curved Zirconia Surfaces. JOURNAL OF DENTAL RESEARCH. 2010;89(Spec Iss B): 4005. (**www.dentalresearch.org**).

***4004 ZIRCONIA MICROTEXTURED SURFACES USING SOFT LITHOGRAPHY***

http://iadr.confex.com/iadr/2010barce/webprogram/Paper140650.html

**PELÁEZ-VARGAS A** [1], GALLEGO-PÉREZ D [2], HIGUITA-CASTRO N [2], HANSFORD DJ [2], and MONTEIRO FJ [3]

[1] INEB/ Instituto de Engenharia Biomédica - Divisão de Biomateriais and FEUP - Universidade do Porto, Porto, Portugal,
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Lithography is the most studied technique of microfabrication to modify hard biomaterial surfaces to introduce ordered textures. However, this set of techniques shows limitations in micro-stamping curved zirconia surfaces.

PDMS microstamping combined with silica sol-gel allows for an interesting alternative for dental implants. Objectives: The purpose of this work was to develop a molding technique based on soft lithography to produce anisotropic microtextured silica thin films on curved zirconia surfaces.

Methods: Yttria stabilized zirconia cylindrical shaped substrates were produced using a standard process. A hybrid silica sol was obtained by sol-gel chemistry using TEOS and MTES precursors. A master model was generated, using a photolithography standard process. Subsequently, a PDMS negative mold was obtained by spinning.

Silica was stamped by pressing PDMS mold on the substrates. Subsequently, samples were dried and sintered, following a thermal cycle that included heating at 500ºC for 60 min with a heating rate of 5ºC/min. SEM/EDS was used to characterize the resulting surfaces.

Results: 3D-silica micropatterned films were obtained on curved surfaces partially corresponding to cylindrical side walls. Preliminary results showed textured surfaces that included pillar micropattened features isotropically distributed with 20 μm interspacing (Figure 35).

Stamping pressure had a critical influence on the resulting micropatterned coatings, with an optimal pressure interval being present, as very low pressures induced excessive film thickness and cracking, while very high pressures led to mold and features deformations.

Conclusions: Soft lithography and sol-gel chemistry combination allows to produce microtextured surfaces on curved substrates zirconia-based.

FCT grant FCT/SFRH/BD/36220/2007, Project Bonamidi PTDC/CTM/100120/2008 and AFOSRMURI F49620-03-1-0421 are acknowledged.

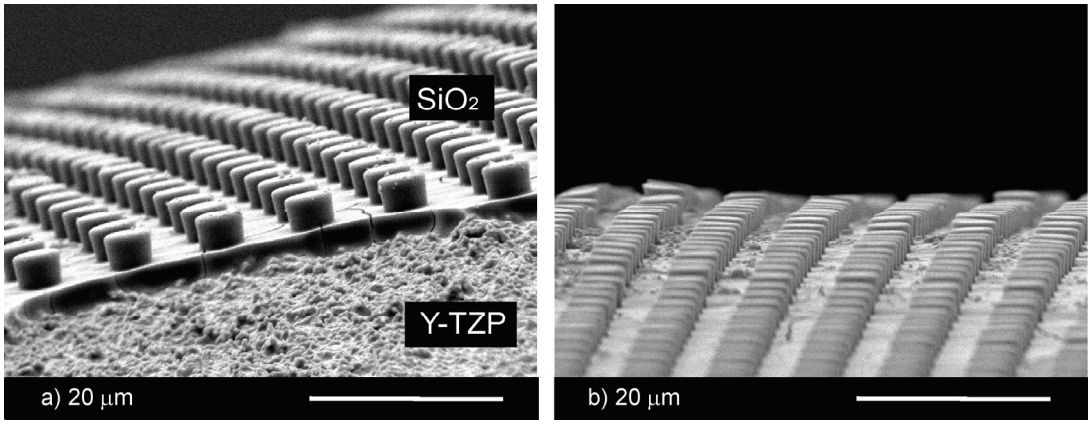



Figure 35. SEM images from micropillared silica coatings applied on Y-TZP cylinder surfaces

## Abstract 7. PORTLAND PARTICULATED SILICA COATINGS – COMPARISON BETWEEN TWO COATING TECHNIQUES

**PELÁEZ-VARGAS A**, HIGUITA-CASTRO N, GALLEGO-PÉREZ D, HANSFORD DJ, and MONTEIRO FJ. Portland Particulated Silica Coatings – Comparison Between Two Coating Techniques. JOURNAL OF DENTAL RESEARCH. 2010;89(Spec Iss B):4006. (**www.dentalresearch.org**).

***4006 PORTLAND PARTICULATED SILICA COATINGS – COMPARISON BETWEEN TWO COATING TECHNIQUES***

http://iadr.confex.com/iadr/2010barce/webprogram/Paper140669.html

**PELÁEZ-VARGAS A** [1], HIGUITA-CASTRO N [2], GALLEGO-PÉREZ D [2], HANSFORD DJ [2], and MONTEIRO FJ [3].

[1]INEB/ Instituto de Engenharia Biomédica - Divisão de Biomateriais and FEUP - Universidade do Porto, Porto, Portugal.
[2]The Ohio State University, Columbus, OH, USA.
[3]INEB/ Instituto de Engenharia Biomédica - Divisão de Biomateriais and FEUP - DEMM - Universidade do Porto, Porto, Portugal.

Bioactive coatings for dental implants have used many different techniques. However, often these produce coatings showing disadvantages such as adhesive/cohesive failure and high temperature/vacuum requirements. Sol-gel technology allows to produce thin films in a flexible and inexpensive manner. Spin, dip and jet coating are amongst the most common techniques used with sol-gel films.

Objectives: To compare spin and dip coatings methods to produce silica thin films containing carbonated Portland cement particles for dental implants.

Methods: Hybrid silica sol was prepared using two silica precursors (TEOS and MTES). Carbonated Portland cement microparticles were prepared by a process that involved milling, floating and drying. Non-carbonated Portland cement particles were used as control. A metastable solution of bioactive particles was prepared and applied using Spin and Dip Coater devices. Characterization was based on SEM/EDS and profilometry.

Results: A transparent silica sol was produced. Addition of non-carbonated Portland cement particles produced a rapid gelation of the suspension

inhibiting film preparation. Carbonated Portland cement particles suspension produced crack-free homogeneous coatings (Figure 36).

Comparisons between both techniques showed that Spin coating allowed for better control of coating thickness in the absence of particles, but these, when present, were more homogeneously distributed when using dip coating technique.

Conclusions: Portland cement particulated silica coatings were produced by dip coating and spin coating. Dip coating deposition techniques avoided the aggregation and heterogeneous distribution of cement particles throughout the films.

FCT grant FCT/SFRH/BD/36220/2007, Project Bonamidi PTDC/ CTM/100120/2008 and AFOSRMURI F49620-03-1-0421 are acknowledged.

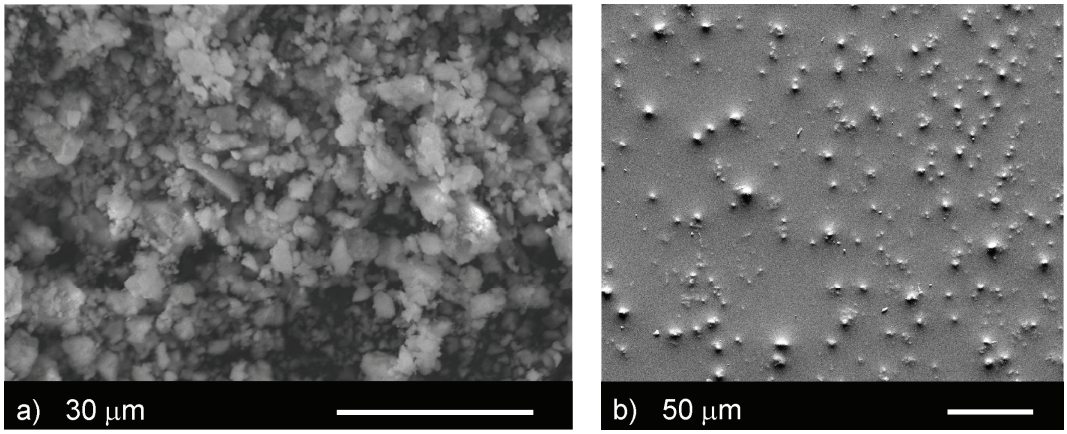



Figure 36. SEM images of Portland Cement powders (a) and Silica Coating modified with Portland Cement Particles.

## Abstract 8. NANOPARTICULATED HA/SILICA THIN FILMS ON ZIRCONIA SURFACES

**PELÁEZ-VARGAS A**, A.H. DE AZA, P. PENA, and MONTEIRO FJ. Nanoparticulated HA/silica thin films on Zirconia surfaces. JOURNAL OF DENTAL RESEARCH. 2010;89(Spec Iss B):1390. (**www.dentalresearch.org**).

***1390 NANOPARTICULATED HA/SILICA THIN FILMS ON ZIRCONIA SURFACES***

http://iadr.confex.com/iadr/2010barce/webprogram/Paper140699.html

**PELÁEZ-VARGAS A** [1], DE AZA AH [2], PENA P [1], and MONTEIRO FJ [3].

[1]INEB/ Instituto de Engenharia Biomédica - Divisão de Biomateriais and FEUP - Universidade do Porto, Porto, Portugal.
[2]Consejo Superior de Investigaciones Científicas - Instituto de Cerámica y Vidrio, Madrid, Spain.
[3]INEB/ Instituto de Engenharia Biomédica - Divisão de Biomateriais and FEUP - DEMM - Universidade do Porto, Porto, Portugal.

Yttria stabilized zirconia has suffered a rapid expansion in dental applications. It covers the range from single anterior crowns and posterior bridges, to implant supported superstructures. The use of zirconia in dental implants has created new challenges for surface modification approaches to improve osseointegration.

Objectives: The aim of this work was to develop silica thin films containing HA nanoparticles aggregates on zirconia surfaces.

Methods: Silica hybrid sol was prepared using two alkoxides (TEOS and MTES), under acid catalysis. Commercially available HA nanoparticles agglomerates were added as bioactive agents to produce a metastable solution with several percentages of solids.

Dip coating was used to apply the nanoHA silica solution onto zirconia-based discs. Sintering was conducted at 500ºC for 60 min with a heating slope of 5ºC/min. Chemical and morphological characterization was done using SEM/EDS.

Results: A metastable nanoHa/silica solution was successfully applied on zirconia-based discs by dip coating technique.

Extraction speed used in the Dip Coater influenced the final quality of the coatings. Homogeneous and crack-free thin films were produced, containing randomly distributed nanoHA particles, when using slower speed (40 mm/min).

For faster extraction speeds cracking of coatings took place due to overgrowth of film, exceeding the sol-gel uncracking critical thickness of the coated layer (Figure 37).

Conclusions: Sol-gel technology has shown to be an adequate technique to produce nanoparticulated HA aggregates/silica thin films.

Slower extraction rates produced more homogeneous and thinner films.

FCT/SFRH/BD/36220/2007 grant and Projects Bonamidi PTDC/CTM/100120/2008 and CRUP E46-09 and Fluidinova S.A. are acknowledged.

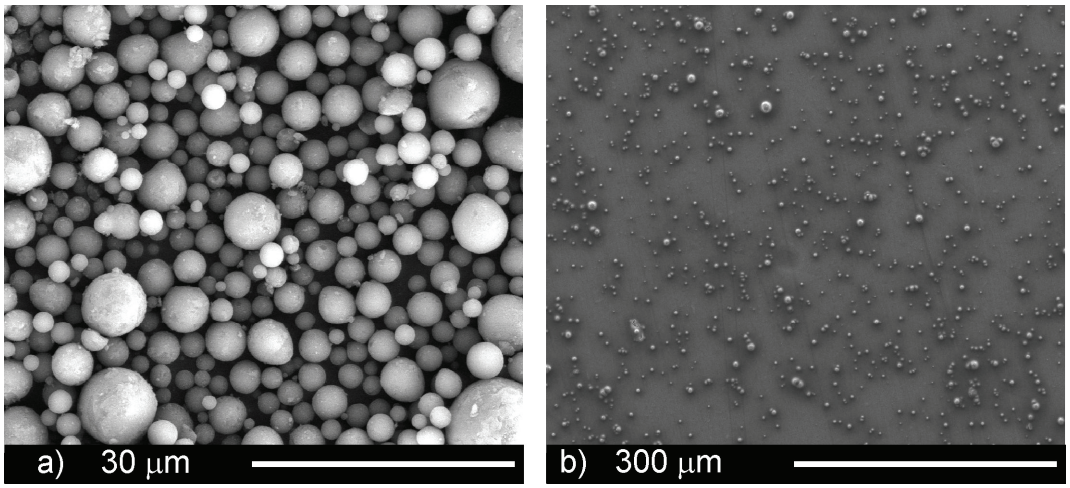



Figure 37. SEM images of micro-agregates of nanohydroxyapatite (a) and Silica Coating modified with these bioactive particles.

## Abstract 9. *S. mutans* AND OSTEOBLAST ADHESION ON SILICA COATINGS

**PELÁEZ-VARGAS A**, FERNANDES MH, FERRAZ MP, MONTEIRO FJ. S. mutans and osteoblast adhesion on silica coatings. JOURNAL OF DENTAL RESEARCH. 2009;88 (Spec Iss A):2551. (**www.dentalresearch.org**).

*2551 S. mutans AND OSTEOBLAST ADHESION ON SILICA COATINGS*

**http://iadr.confex.com/iadr/2009miami/webprogram/Paper120093.html**

**PELÁEZ-VARGAS A** [1], FERNANDES MH [2], M.P. FERRAZ [3] and MONTEIRO FJ [4].

[1] INEB – Instituto de Engenharia Biomedica, Universidade do Porto, FEUP, Porto, Portugal and Universidad CES, Medellín, Colombia.
[2] Laboratório de Farmacologia e Biocompatibilidade Celular, Faculdade de Medicina Dentária, Universidade do Porto, Porto, Portugal.
[3] Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.
[4] INEB - Divisão de Biomateriais and Departamento de Engenharia Metalúrgica e Materiais, FEUP, Universidade do Porto, Porto, Portugal.

Introduction: Silica coatings on dental implants might be a promising approach to reduce bacterial colonization and increase osseointegration due to their flexibility to modify surface physical and chemical features.

Objectives: To evaluate adhesion of S. mutans and Human Bone Marrow (HBM) cells cultured on silica thin films produced by sol-gel.

Methods: Glass cover slips were coated with $SiO_2$ thin films by sol-gel route under acid catalysis in single stage. The coatings were applied using a Spin Coater, followed by sintering at 450ºC for 60 min and autoclave sterilization. Thickness and surface wettability were evaluated using SEM and the sessile method of contact angles respectively (Figure 38). Glass cover slips were used as control.

S. mutans were grown under standard protocol for 72 h at 37ºC. Bacterial cell concentration was adjusted (0.5 MacFarland at A660) and bacteria were cultured on control and $SiO_2$-coated samples for 4 hours. Fluorescence microscopy was used to evaluate the area colonized by S. mutans.

HBM (Human Bone Marrow) cells were cultured according to a

standardized protocol for 4h and 24h. Cell morphology was observed by Scannning Electron mycroscopy.

Results: Homogeneous and defectless flat silica thin films with thicknesses of 1,35(0.06)µm were produced. The contact angles were 66.3º(±1.4º) and 19.5º(±1.7º) for $SiO_2$-coated and control samples, respectively.

S. mutans area colonized was six times higher on the control surface than on the silica coating surface at 4h of incubation. HBM cells cultured on silica thin film coatings showed similar adhesion and spreading compared to the control group.

Conclusions: These preliminary studies showed that Silica thin film coating exhibited a hydrophobic surface with reduced S. mutans colonization and similar HBM adhesion when compared with non-coated samples, suggesting a potential approach to modulate cell surface interaction.

Acknowledgment: The PhD grant FCT/SFRH/BD/36220/2007 is acknowledged.

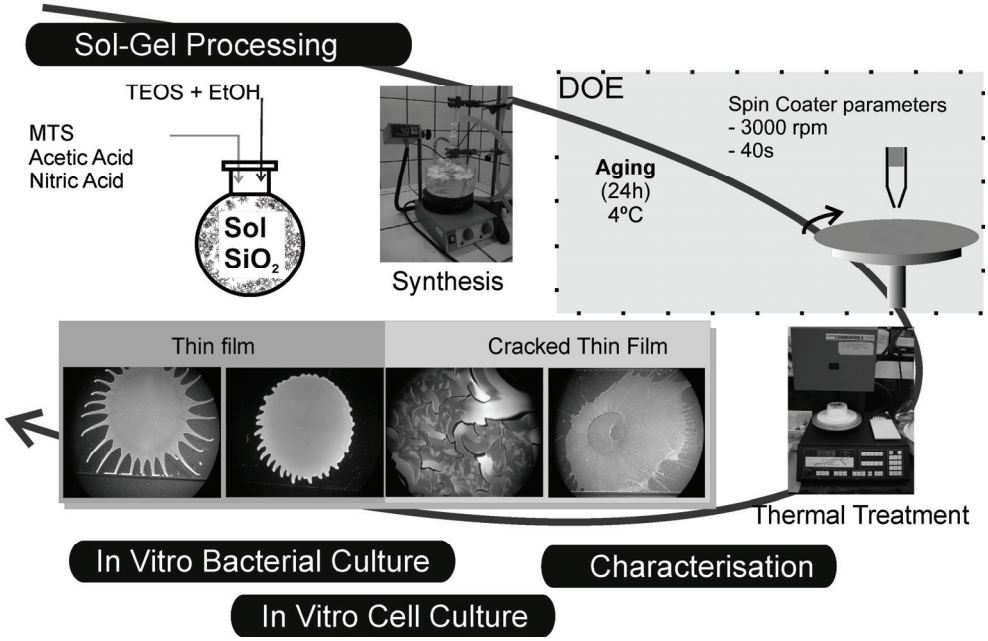



Figure 38. Sol-gel processing to produce optimized silica coatings. These were evaluated in terms of osteoblast behavior and St. mutans adherence.

## Abstract 10. PRODUCCIÓN DE TEXTURAS POR MICROESTAMPACIÓN SOBRE CERÁMICAS DENTALES

**PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, HIGUITA-CASTRO N, FERNANDES MH, PENA P, DE AZA AH, HANSFORD D, MONTEIRO FJ. 50 Congreso de la SECV- Sociedad Española de Ceramica y Vidrio. Madrid (Spain). 27 a 29 Outubro 2010

***PRODUCCIÓN DE TEXTURAS POR MICROESTAMPACIÓN SOBRE CERÁMICAS DENTALES***

**PELÁEZ-VARGAS A** [1, 2, 3], D. GALLEGO-PÉREZ D [3], HIGUITA-CASTRO N[3], FERNANDES MH [4], PENA P [5], DE AZA AH [5], HANSFORD D [3], MONTEIRO FJ[1, 2]

[1] INEB – Instituto Nacional de Engenharia Biomédica. Porto, Portugal.
[2] DEMM – Departamento de Engenharia Metalúrgica e Materiais, FEUP - Faculdade de Engenharia, Universidade do Porto. Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal.
[3] Department of Biomedical Engineering, The Ohio State University. Columbus, Ohio, USA
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*La litografía es la técnica más frecuentemente utilizada para producir texturas sobre superficies para aplicaciones biomédicas. Sin embargo, se conoce poco sobre sus posibilidades para modificar la superficie de biomateriales dentales duros, y el impacto de está tecnología sobre el comportamiento in Vitro de células de la cavidad oral. El presente trabajo fue diseñado con el objetivo de producir texturas sobre superficies de biomateriales dentales utilizando microestampación y evaluar los efectos de la textura sobre comportamiento in Vitro de fibroblastos gingivales y osteoblastos. Inicialmente se utilizó la fotolitografía como técnica para producir un modelo maestro que contenía geometrías regulares e irregulares entre un rango de escala entre 200 micrómetros y 500 nanómetros. Posteriormente, se creo un molde negativo en PDMS que sirvió para dos propósitos: 1) Microestampar recubrimientos vítreos preparados por sol-gel sobre discos basados en biomateriales dentales como circona, porcelana feldespática, alumina y vidrios, como grupo control. 2) Producir texturas ordenadas sobre superficies de cerámicas dentales sin el uso de recubrimientos. Finalmente, se realizó una caracterización de las superficies utilizando microscopia electrónica de barrido, y una evaluación temprana de los efectos sobre el comportamiento celular in Vitro de fibroblastos y osteoblastos. Los resultados de esté trabajo mostraron que: 1) es posible*

*microestampar recubrimientos vítreos con microtexturas sobre diferentes materiales dentales, incluso en escalas submicrométricas, así como producir directamente superficies cerámicas con microtexturas. 2) Las superficies con microtexturas separadas por 10 micrómetros, mostraron los efectos tempranos más claros sobre el comportamiento biológico in Vitro.*




Figure 39. Partial cover image from abstracts book

# Manuscript 10. BACTERIAL ADHESION AND OSTEOBLASTIC BEHAVIOR ON SILICA COATINGS OPTIMIZED USING STATISTICAL EXPERIMENTAL DESIGN

**ALEJANDRO PELÁEZ-VARGAS** , JOSÉ HERNEY RAMÍREZ-FRANCO, MARÍA HELENA FERNANDES, MARIA PIA FERRAZ, FERNANDO J. MONTEIRO

***BACTERIAL ADHESION AND OSTEOBLASTIC BEHAVIOR ON SILICA COATINGS OPTIMIZED USING STATISTICAL EXPERIMENTAL DESIGN***

**PELÁEZ-VARGAS A** [1, 2], RAMÍREZ-FRANCO JH, [3] FERNANDES MH, [4] FERRAZ MP, [1, 5] MONTEIRO FJ [1, 2]

[1] INEB-Instituto de Engenharia Biomédica, Divisão de Biomateriais, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal.
[2] Universidade do Porto, Faculdade de Engenharia, Dep. Eng. Metalúrgica e Materiais, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal.
[3] Universidad Nacional de Colombia. Departamento de Ingeniería Química. Carrera 30 # 45-03, Ciudad Universitaria. Bogotá, Colombia.
[4] Laboratório de Farmacologia e Biocompatibilidade Celular, Faculdade de Medicina Dentária, Universidade do Porto, Rua Dr. Manuel Pereira da Silva, 4200-393 Porto, Portugal.
[5] Faculdade de Ciências da Saúde. Universidade Fernando Pessoa. Porto, Portugal.

*Rejected in Journal Chemistry of Materials and Colloids and Surfaces B: Biointerfaces. Some changes are necessaries to submit for a new journal.*

A factorial fractionate statistical design was conducted to optimize the thickness of Silica thin film coatings produced by sol-gel technology and applied by spin coating. Thickness ad-hoc model included variables defined as rotational speed, delay time and aging time. Multivariate experimental

design allowed to develop an ad-hoc mathematical model to predict the thickness of silica coatings conditioned by the solution aging, delay time and speed rotation. Statistical experimental design was a good tool to develop and optimize silica coatings with a reduced amount of experiments. These optimized hydrophobic coatings were characterized in terms of biological behavior when in contact with *S. mutans* and Human Bone Marrow cells.

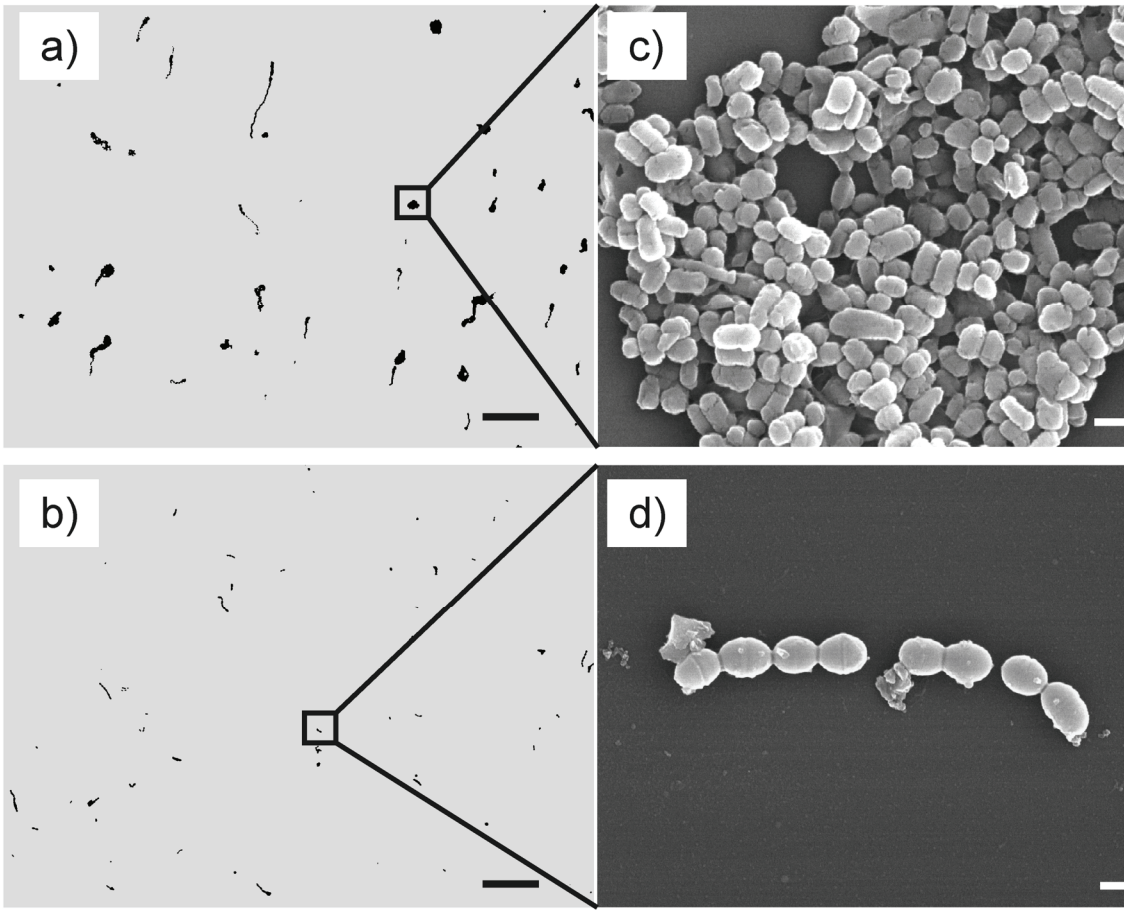



Figure 40. Representative epifluorescence images in inverted gray scale (a,b) and SEM images (c,d) of S. mutans.

Early adhesion at 4h period on glass substrates (a,c) and on silica surfaces(b,d). A lower adherence may be observed on silica surfaces than on glass substrates. Bar scale (a-b = 20 µm and c-d = 1 µm).

A decreased bacterial adherence to the coatings was found when compared to

glass substrate (Figure 40), without cytotoxic effects on Human Bone Marrow osteoblastic cells. Silica coatings can be considered as flexible matrixes to obtain reduced bacterial adherence due to their hydrophobicity and surface smoothness with potential use in dental applications.

# APPENDIX C. CELLULAR BEHAVIOR ON THE PATTERNED SURFACES DEVELOPED

**Overview**

This appendix section is divided in nine abstracts presented in the Annual Meeting of International Association for Dental Research in Toronto (Canada, 2008) and Barcelona (Spain, 2010). As well as, in the Biomedical Engineering Society Annual Meeting (Pittsburg, USA. 2009), the Materials Research Society Fall Meeting & Exhibition (Boston, MA. 2009).

- **Abstracts**

***In the 11th abstract***, entitled *"Shape control and orientation of hbm cells on micropatterned coatings"*. The purpose of this work was to evaluate how surface topography affects the morphology and alignment of Human Bone Marrow (HBM) cells cultured In Vitro on micro-patterned (micro-PGC) and flat glass coatings (FGC) produced by sol-gel processing.. This abstract is referenced on Journal of Dental Research.

***In the 12th abstract***, entitled *"Anisotropic microtextured silica thin films on zirconia"*. The purpose of this study was to develop anisotropic microstructured $SiO_2$ thin film on zirconia surfaces. This abstract is referenced on Journal of Dental Research.

***In the 13th abstract***, entitled *"Microstructured coatings to study the behavior of osteoblast-like cells on hard materials"*. The purpose of this work was to evaluate the adhesion and proliferation of Human Bone Marrow Stem Cells (HBMSCs) on pillar-shape microtextured silica coatings with different interspacing.. This abstract is referenced on BONE.

***In the 14th abstract***, entitled *"Proliferation of mesenchymal stem cells on stamped thim films modified with nanohydroxyapatite particles"*. The purpose of this work was to evaluate the adhesion, proliferation and metabollic activity of Mesenchymal Stem Cells (MSCs) on microfabricated silica thin films modified with nanoHydroxyapatite particles (nanoHA). This abstract is referenced on BONE.

***In the 15th abstract***, entitled *"HGF Adhesion on Modified Surfaces of Zirconia Based Framework Cores"*. The purpose of this work was to compare the adhesion, morphology and proliferation of Human Gingival Fibroblast (HGF) cells cultured on surface modified zirconia based framework cores (ZBFC). This abstract is referenced on the proceedings MRS FALL Meeting 2009.

***In the 16th abstract***, entitled *"Micro-Structured Silica Coatings with HA Particles Using a PDMS Stamp"*. The purpose of this work was to develop a technique that includes silica sol-gel chemistry and soft lithography technology to produce micropatterned silica coatings with hydroxyapatite nanoparticles (nanoHA). This abstract is referenced on the proceedings of MRS FALL Meeting 2009.

***In the 17th abstract***, entitled *"Silica coatings with Portland cement particles for dental implant surfaces based on sol-gel micromolding"*. The purpose of this work was to develop a dual molding technique based in soft lithography to produce micropatterned silica coatings with carbonated Portland cement particles (CPCP). This abstract is referenced on Proceeding of Biomedical Engineering Society 2009.

***In the 18th abstract***, entitled *"Microassembly of Multifunctional Bone Tissue Engineering Scaffolds"*. The purpose of this work was to use a soft

lithography approach to fabricate scaffolds with controlled microstructure and chemistry in layers that are then assembled into a collective scaffold structure. Individual layers were manufactured from various biomedical polymers (PLGA, PCL, PCL/nanohydroxyapatite, PMMA, and PVDF). This abstract is referenced on Proceeding of Biomedical Engineering Society 2009.

***In the 19th abstract***, entitled *"Micro-patterning combined with live-cell imaging: A tool for in vitro studies of dental tissue guided regeneration"*. The purpose of this work was to study the in vitro dynamic behavior of cells derived from human oral tissues (Human Pulp-derived MSCs) on micropatterned hard materials using time-lapse microscopy and digital image analysis. This abstract is referenced on the proceedings of I3S Retreat 2011.

- **Papers**

**11th manuscript**, entitled "*Effects of density of anisotropic micro-stamped silica thin films on guided bone tissue regeneration – in vitro study",* is under revision in Dental Materials (July 2011). This paper studied the differential responses of human osteoblast-like cells to anisotropic silica surface textures with varying densities.

**12th manuscript**, entitled "*Portland cement porous scaffolds for load-bearing bone tissue engineering applications*". This paper was accepted in the Journal of Biomedical Materials Resesearch. Part B - Applied Biomaterials. Due that this is a secondary work made during the period as Visitang Scholar in the Professor. Hansford's Group at The Ohio State University. The information only is presented as abstract. The aim of this paper was to describe the fabrication and characterization of porous scaffolds from modified/cytocompatible Portland cement, for applications in load-bearing BTE.

## Abstract 11. SHAPE CONTROL AND ORIENTATION OF HBM CELLS ON MICROPATTERNED COATINGS

**PELÁEZ-VARGAS A**, FERRELL N, FERNANDES MH, HANSFORD D, MONTEIRO FJ. Shape control and orientation of HBM cells on micropatterned coatings. JOURNAL OF DENTAL RESEARCH. 2008;87(Spec Iss B):3314. (**www.dentalresearch.org**).

*3314 SHAPE CONTROL AND ORIENTATION OF HBM CELLS ON MICROPATTERNED COATINGS*

http://iadr.confex.com/iadr/2008Toronto/techprogram/abstract_106841.htm

**PELÁEZ-VARGAS A** [1], FERRELL N [2], FERNANDES MH [3], HANSFORD DJ [2], and **MONTEIRO FJ** [4].

[1] INEB - Divisão de Biomateriais, FEUP, Universidade do Porto, Porto, Portugal and Universidad CES, Medellín, Colombia.
[2] Department of Biomedical Engineering, The Ohio State University, Columbus, USA.
[3] Laboratório de Farmacologia e Biocompatibilidade Celular, Faculdade de Medicina Dentária, Universidade do Porto, Portugal.
[4] INEB - Divisão de Biomateriais and Departamento de Engenharia Metalúrgica e Materiais, FEUP, Universidade do Porto, Portugal.

Introduction: Combining sol-gel and microfabrication techniques provides an alternative to generate model surfaces to study topographical modulation of cell-biomaterial interactions.

Objectives: To evaluate how surface topography affects the morphology and alignment of Human Bone Marrow (HBM) cells cultured In Vitro on micro-patterned (micro-PGC) and flat glass coatings (FGC) produced by sol-gel processing.

Materials and Methods: Photolithography was used to produce master patterns of parallel lines and pillars with microscale dimensions. A polydimethylsiloxane (PDMS) mold was obtained from the masters by uniformly mixing PDMS (Silastic T-2, Dow Corning, USA) with a curing agent, pouring the mixture onto the masters, degassing, and curing. The mold was used to print $SiO_2$ glass coatings produced by a sol-gel process. The coatings were then sintered and sterilized (Figure 41).

In vitro biological evaluation was conducted using HBM cells, cultured with a standardized protocol for four days. Cell morphology and orientation were observed using scanning electron and optical microscopy.

Results: Flat and MPGC with lines and pillar shaped features with 5µm periodicity and ~3µm height were produced. Cells presented a normal morphology on flat surfaces while slimmer, preferential orientation and more elongated morphologies could be seen on both the line and pillar micro-patterned surfaces.

HBM cells cultured on flat glass coatings showed spreading and more random proliferation as compared to the cells on the micro-patterned glass coatings. Micro-patterned glass coatings with pillar shaped features showed lower orientation control than line shaped micro-patterned glass, but higher than flat glass coatings. A number of cells on the pillar surface presented an interesting and peculiar behavior. Cells stretched long distances, often more than 100µm, in one specific direction to reach and connect with other cells.

Conclusions: These preliminary studies revealed that Micro-PGC controlled the orientation of HBM cells and induced significant changes in their morphology.

Acknowledgment: Program-Alban/E05D050652CO, FCT/SFRH/BD/36220/2007 and FCT/POCI/SAU-BMA/56061/2004.

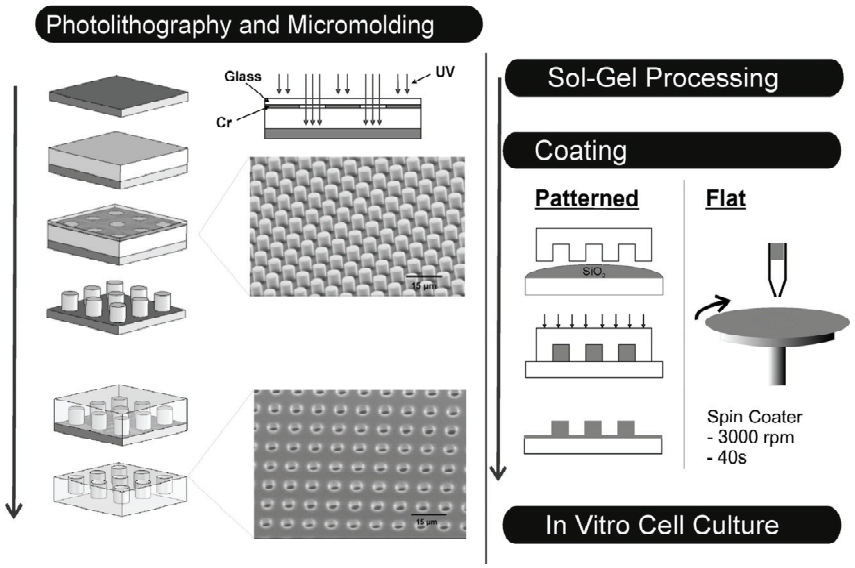



Figure 41. Flow process that include photolithography and micromolding production to stamping silica thin films compared with flat thin film application using Dip Coating

## Abstract 12. ANISOTROPIC MICROTEXTURED SILICA THIN FILMS ON ZIRCONIA

**PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, FERNANDES MH, HANSFORD DJ, and MONTEIRO FJ. Anisotropic microtextured silica thin films on zirconia. JOURNAL OF DENTAL RESEARCH. 2010;89(Spec Iss B):1391. (**www.dentalresearch.org**).

### *1391 ANISOTROPIC MICROTEXTURED SILICA THIN FILMS ON ZIRCONIA*

**http://iadr.confex.com/iadr/2010barce/webprogram/Paper140713.html**

**PELÁEZ-VARGAS A** [1], GALLEGO-PÉREZ D [2], FERNANDES MH [3], HANSFORD DJ [2], and MONTEIRO FJ [4].

[1] INEB/ Instituto de Engenharia Biomédica - Divisão de Biomateriais and FEUP - Universidade do Porto, Porto, Portugal,
[2] The Ohio State University, Columbus, (OH).USA.
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Guided tissue regeneration is an old challenge in dentistry. Several strategies have been developed to be used in polymers. However, few solutions for hard materials are available. Microfabrication technology provides alternatives to producing well organized microtextured surfaces, and when it is combined with sol-gel technology, it may help to modulate cell behavior on Yttria Stabilized Zirconia surfaces for dental implants.

Objectives: The purpose of this study was to develop anisotropic microstructured $SiO_2$ thin film on zirconia surfaces.

Methods: Ytria stabilized zirconia (Y-TZP) discs were produced, using a standard process that included pressing, pre-sintering, soft milling, sintering and polishing. Silica sol was generated by sol-gel reaction.

A polydimethylsiloxane (PDMS) mold was obtained from the masters produced by photolithography. The mold was used to stamp silica,

producing micropatterned thin films with an isotropic texture.

Characterization of micropatterned thin films was conducted by SEM/EDS. In vitro biological evaluation was conducted using Human Gingival Fibroblastic cells, cultured with a standardized protocol for 4 and 24h. Alignment was studied using Fluorescence microscopy.

Results: Optimum conditions for anisotropic micropatterned silica coatings on Y-TZP substrates were obtained with a spacing of 10 μm.

Notorious alignment of Human Gingival Fibroblast could be detected after 24h of cell cultures on anisotropic micropatterned silica coatings, as opposed to more ramdomised cell orientation after just 4h cell culture.

Conclusions: These preliminary studies revealed that anisotropic micropatterned silica coatings may produce controlled orientation of Human Gingival Fibroblast (Figure 42).

FCT grant FCT/SFRH/BD/36220/2007, Project Bonamidi PTDC/CTM/100120/2008 and AFOSRMURI F49620-03-1-0421 are acknowledged.

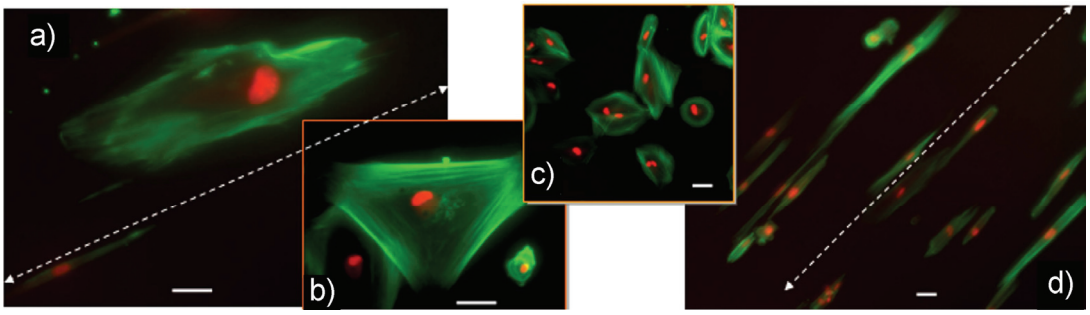



Figure 42. Fluorescence images from Human Gingival Fibroblast on anisotropic micropatterned and flat silica thin films produced on Y-TZP.

# Abstract 13. MICROSTRUCTURED COATINGS TO STUDY THE BEHAVIOR OF OSTEOBLAST-LIKE CELLS ON HARD MATERIALS

**PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D D, FERNANDES MH, HANSFORD DJ and MONTEIRO FJ. Microstructured coatings to study the behavior of osteoblast-like cells on hard materials. BONE. 2011;48(Suppl 2):S105-S106.

***PP007-S MICROSTRUCTURED COATINGS TO STUDY THE BEHAVIOR OF OSTEOBLAST-LIKE CELLS ON HARD MATERIALS***

www.sciencedirect.com

**PELÁEZ-VARGAS A**[1, ,2] , GALLEGO-PÉREZ D [3], FERNANDES MH [4], HANSFORD DJ [3], and MONTEIRO FJ [1, 2].

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[3] Department of Biomedical Engineering. The Ohio State University, Columbus, OH,
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Introduction: Soft lithography has been successfully used to study cell-material interactions on microtextured polymeric surfaces. However, the information available about osteoblast-like cell behavior on micropatterned hard surfaces is limited due to the challenges related to the processing of hard materials. The combination of sol-gel and soft lithography could present a simple alternative to overcome these obstacles.

Objectives: The aim of the work was to evaluate the adhesion and proliferation of Human Bone Marrow Stem Cells (HBMSCs) on pillar-shape microtextured silica coatings with different interspacing.

Methods: UV-photolithography was used to produce a pillar-shaped master model with different interspacing (5, 10, 20 and 45 μm). PDMS negative molds were produced from the master model. These molds were used to create isotropic microstructured silica sol-gel coatings on hard substrates. After sintering, the coatings were sterilized and used to evaluate the adhesion, proliferation/metabolic activity and ALP activity of HBMSCs at different time points (1, 7, 14, 21). TCPS was used as a control group. Morphological characterization of the cells was conducted using SEM/EDS and fluorescence microscopy. The Alamar Blue and ALP kinetics assays were used to evaluate the metabolic activity.

Results: The microscale geometry from the master was successfully transferred onto the silica coatings via the PDMS mold. Pillar-shaped coating showed features of ~5 µm diameter with 5, 10, 20 and 45 µm interspacing. Morphological analysis showed that HBMSCs on pillars with 5 and 10 µm interspacing exhibited abundant cytoplasm extensions, which tended to decrease when pillar interspacing was increased (Figure 43). The cells showed preferential adhesion to the top surface of the pillars (5 and 10 µm interspacing). The alamar Blue assay showed that the cells were viable at all time points, and that the highest viability values at Day 1 were found for the 5 µm interspacing features. The activity of ALP was statistically higher ($p<0.05$) for the micropillar surface with 20 µm interspacing when compared to the other spacings and control group at Day 21.

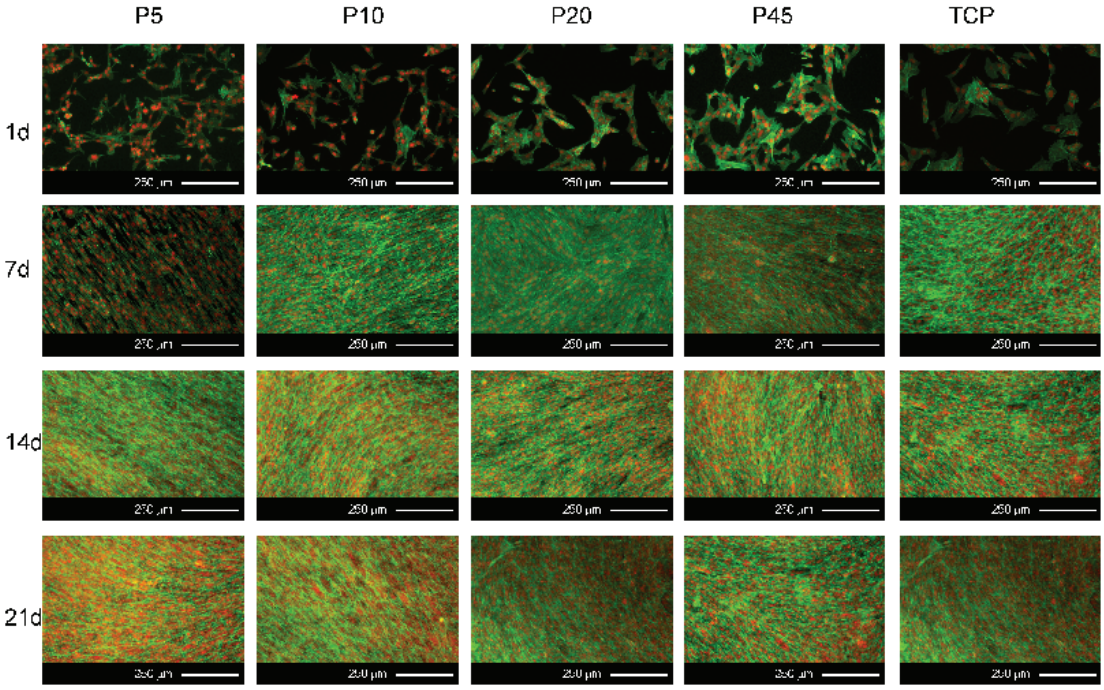



Figure 43. Fluorescence images of HBMSCs on pillar-shape microfabricated silica coatings.

P5=5 µm, P10=10 µm, P=20 µm and P45=45 µm of interspacing and control (TCP) for different time points (1, 7, 14 and 21 days).

Conclusions: Preliminary results show that micropillars on hard materials may induce higher metabolic activity at early and advanced cultures stages.

FCT/SFRH/BD/36220/2007 grant and Projects Bonamidi PTDC/CTM/100120/2008 and CRUP E46-09 are acknowledged.

## Abstract 14. PROLIFERATION OF MESENCHYMAL STEM CELLS ON STAMPED THIM FILMS MODIFIED WITH NANOHYDROXYAPATITE PARTICLES

CARVALHO A, **PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, FERNANDES MH, HANSFORD DJ and MONTEIRO FJ. Proliferation of mesenchymal stem cells on stamped thim films modified with nanohydroxyapatite particles. BONE. 2011;48(Suppl 2):S106.

***PP010-S PROLIFERATION OF MESENCHYMAL STEM CELLS ON STAMPED THIM FILMS MODIFIED WITH NANOHYDROXYAPATITE PARTICLES***

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Introduction: Guided tissue regeneration for dentistry is a therapy focused on there-growth of bone to fill infra-bony defects produced by periodontal diseases. The ability to add microtexture to implant surfaces through a simple process, such as sol-gel, can aid in the guiding of tissue alignment through contact guidance.

Objectives: The aim of the work was to evaluate the adhesion, proliferation and metabollic activity of Mesenchymal Stem Cells (MSCs) on microfabricated silica thin films modified with nanoHydroxyapatite particles (nanoHA).

Methods: Microtextured silica thin films were produced using a microstamping technique that is a combination of soft lithography and sol-gel processing. Nanoscale hydroxyapatite particles were introduced into the sol for incorporation into the thin films at three difference weight ratios (0, 1, and 5%). Materials characterization of the films was conducted using SEM/EDS.
Cell cultures of pulp-derived MSCs were conducted under standardized conditions at three time-points (1, 7 and 14 days). TCPS was used as control

group for all experiments. Morphological analysis was conducted via fluorescence (staining for F-actin and nuclei) and electron microscopy. Metabollic activity was conducted using Resazurin and ALP assays.

Results: Anisotropic silica micropatterned thin films containing different percentages of nanoHA particles were successfully produced. Line-shaped micropatterns faithfully reproduced the mold features with ~5 µm width and ~10 µm interspacing (Figure 44).

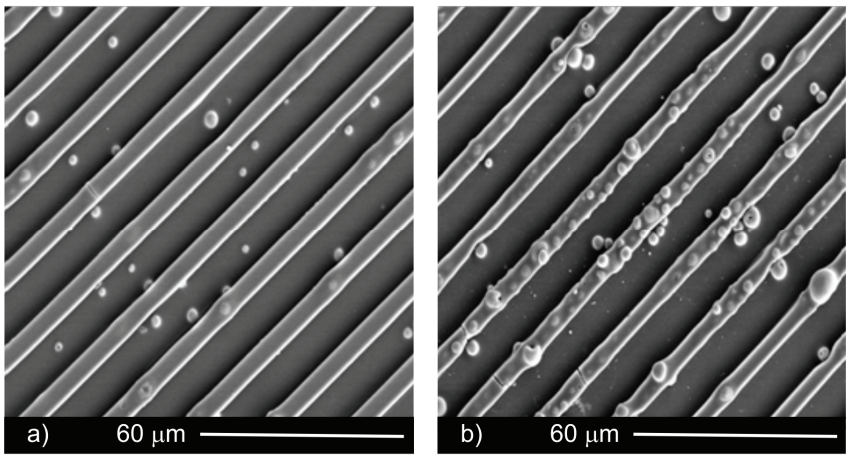



Figure 44. Anisotropic silica micropatterned thin films containing different percentages of nanoHA particles.

Line-shaped micropatterns faithfully reproducing the mold features with ~5 µm width and ~10 µm interspacing, where: $SiO_2$+ 1% nanoHA (right) and $SiO_2$+ 5% nanoHA (left). Bar scale = 60 µm.

MSCs showed alignment following the texture of micropatterned surfaces starting at Day 1. This behavior increased in the subsequent time points on all the microtextured surfaces, compared to the TCPS control group. Intragroup analysis using Resuzarin assay showed increased cell numbers at all time points. Intergroups analysis showed that MSCs exhibited increased proliferation on the 5% nanoHA group micropatterns compared to the 0% and 1% groups ($p<0.05$) at Day 7. MSCs on all micropatterned surfaces showed a significantly higher ALP activity ($p<0.05$) when compared to the flat surface of control group at Day 14.

Conclusions: The preliminary results show that all the micropatterned thin films induced the alignment of MSCs and led to improved cellular responses, which were maintained for prolonged periods of time.

FCT/SFRH/BD/36220/2007 grant and Projects Bonamidi PTDC/CTM/100120/2008 and CRUP E46-09 are acknowledged.

# Manuscript 11. EFFECTS OF DENSITY OF ANISOTROPIC MICRO-STAMPED SILICA THIN FILMS ON GUIDED BONE TISSUE REGENERATION – IN VITRO STUDY

**PELÁEZ-VARGAS A**.[1, 2, 3*], GALLEGO-PÉREZ D.[3*], FERNANDES MH.[4], HANSFORD DJ.[3], MONTEIRO FJ[1, 2].

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*Submitted to Dental Materials. July 2011*

## ABSTRACT

Although titanium implants are the gold standard in dentistry, the growing demand for better implant aesthetics has led to increased research on the development of all ceramic dental implants which, albeit structurally strong, possess lower tissue integrative capabilities compared to their titanium counterparts. We have proposed the use of microtextured thin films with more bioactive/versatile chemistries as a way to improve tissue integrability

of ceramic implants. The differential responses of human osteoblast like cells to anisotropic silica microtextures with varying densities, produced via a combination of sol-gel and soft lithography processing, were evaluated in terms of alignment, elongation, overall cellular activity, and the expression/activity levels of alkaline phosphatase (ALP). The results indicated that the microtextures induced guided cell alignment, elongation and colonization on the surface. Cells cultured on denser micropatterns exhibited increased activity (t=14, 21 days), as determined by the alamar blue method. The early (t=7 days) expression/activity levels of ALP released into the medium were found to be significantly higher only on the least dense micropattern. Altogether, our results suggest that microstructured silica thin films could be used to guide and enhance peri-implant cell/tissue responses, thus potentially improving tissue integration of ceramic dental implants.

## 1. INTRODUCTION

Titanium-based dental implants with ceramic crowns have been widely used for the treatment of partial/full edentulism. The success of this metal from a functional point of view relies on its cytocompatibility, osseointegrability, and strength (Albrektsson, Branemark *et al.*, 1981). Previous studies have shown however, that metals, including titanium, could cause non-specific immunomodulation and autoimmune responses (Lalor, Revell *et al.*, 1991). In addition, considering the growing demand for better dental aesthetics, titanium implants still pose a challenge for clinicians, especially with anterior segment implants, as the grayish color of the metal could show through the thin peri-implant mucosa. Moreover, the implant head could become visible overtime due to peri-implant soft tissue recessions, which are often inevitable in patients with a thin gingival biotype unless supplementary treatments such as connective osseous grafts are implemented (Tan and

Saltzman, 2004). Alternatively, ceramics such as zirconia-toughened alumina (ZTA), and yttria-stabilized tetragonal zirconia (Y-TZP), present excellent fracture resistance, and improved aesthetics compared to metals. This has led to increased interest in the development of all ceramic dental implants to fulfill the aesthetic demands not met by titanium implants (Chevalier, 2006; Ahmad, 2010; Pelaez-Vargas, Gallego-Perez *et al.*, 2010).

Although ZTA and Y-TZP have the potential to offer adequate aesthetic and structural properties for the development of all-ceramic dental implants, the tissue-integrative capabilities of these two ceramics are far inferior compared to titanium. Dental implants are interfaced with soft tissue at the neck, and bone tissue at the shaft (Heil, Reifferscheid *et al.*, 1996). A strong and stable bond between soft/hard tissue and the implant is crucial not only for the purpose of early stability, but also to prevent bacterial invasion and the occurrence of peri-implantitis, which is responsible for up to 30% of the cases of long-term (≥10 years) implant failure (Albrektsson, Dahl *et al.*, 1988; Adell, Eriksson *et al.*, 1990; Heil, Reifferscheid *et al.*, 1996; Binon, 2000; Klinge, Hultin *et al.*, 2005; Roos-Jansaker, Lindahl *et al.*, 2006).

Besides the ability to self-passivate, which protects the metal from corrosion and increases its bioactivity, three decades of surface engineering on titanium implants have resulted in the development of numerous surface modification technologies to improve the degree of tissue integration. Of these, techniques that aim at introducing a surface micro-nanotopography have been consistently demonstrated to have a positive effect in this process (Chehroudi, Gould *et al.*, 1989; Gotfredsen, Hjorting-Hansen *et al.*, 1990; Buser, Schenk *et al.*, 1991; Heil, Reifferscheid *et al.*, 1996; Chehroudi and Brunette, 2002; Mendonça, Mendonça *et al.*, 2008; Pelaez-Vargas, Gallego-Perez *et al.*, 2011b).

This knowledge however, cannot be directly translated into the modification of ceramic implants, as most of these techniques could cause surface defects and mechanical failure, or could result in leaching of the stabilizers (e.g. Yttria), aging, and ultimately implant failure (Abron, Hopfensperger *et al.,* 2001; Ogawa and Nishimura, 2003; Klinge, Hultin *et al.,* 2005; Grew, Ricci *et al.,* 2008; Pelaez-Vargas, Gallego-Perez *et al.,* 2010). Therefore, new techniques need to be developed in order to take full advantage of the potential benefits offered by ceramic dental implants.

One potential approach to improving the tissue-integrative capabilities of ceramic implants is the use of thin films with more bioactive and versatile chemistries. We recently demonstrated that ceramic glasses such as silica can be used to modify the surface of Y-TZP dental ceramics via sol-gel processing. Moreover, when applied in combination with soft lithography-based techniques, the silica overcoat can be imparted with an ordered surface microtexture that can be used to modulate cell responses, and potentially improve tissue integration *in vivo* (Pelaez-Vargas, Gallego-Perez *et al.,* 2010). However, optimum engineering of such surfaces for the envisioned application still requires further evaluation of the effects of various topographies and chemistries among other things. This paper studied the differential responses of human osteoblast-like cells to anisotropic silica surface textures with varying densities.

## 2. MATERIALS AND METHODS

### a. Preparation of isotropically and anisotropically microtextured silica thin films

A silica solution was produced via sol-gel processing as described elsewhere (Gallardo, Galliano *et al.,* 2000a; Garcia, Cere *et al.,* 2004; Pelaez-

Vargas, Gallego-Perez *et al.*, 2010). Tetraethylorthosilicate (TEOS, Aldrich) and methyltriethoxisilane (MTES, Aldrich) were used as precursors at a 4:6 molar ratio. Alcohol and nitric/acetic acids served as solvent and catalysts, respectively. The solution was pre-aged for 24h at 4°C and then drop cast on glass coverslips. Thin film microtexturing was conducted via imprinting using previously patterned polydimethylsiloxane (PDMS, Silastic T-2, Dow Corning, USA) stamps, molded from photolithographically-fabricated silicon masters (Figure 46a). All coatings were then sintered at 500°C for 60 minutes. The anisotropic topographies consisted 5x5 μm lines spaced by 5, 10, 20 and 45 μm (edge-edge). Control/flat silica films were prepared by spin coating the solution on glass coverslips at 3000 rpm for 45 seconds. Film characterization was done by scanning electron microscopy (SEM).

**b. Human osteoblast-like cell cultures**

Human osteoblast-like cells (SaOS-2, ATCC, USA) were regularly cultured in α-minimum essential medium (Sigma, USA) supplemented with 10% fetal bovine serum (ATCC, USA), 50 μg/ml ascorbic acid (Sigma, USA) 1% penicillin-streptomycin (Sigma, USA) and 1% gentamicin-amphotericin (Invitrogen, USA). SaOS-2 cells been reported to exhibit many characteristics of well differentiated osteoblasts including the expression of a number of bone-related proteins and cAMP responsiveness to PTH stimulation (McQuillan, Richardson *et al.*, 1995; Hughes FJ, 1998).

The cells were seeded on the anisotropic silica textures at a density of $2x10^4$ cells/$cm^2$. At day 4, the medium was supplemented with 10nM dexamethasone (Sigma, USA), and 10 mM β-glycerol phosphate (Sigma, USA) to induce a more osteogenic phenotype. The cells were further cultured for up to 21 days. Cell cultures on tissue culture-treated polystyrene (TCPS) and flat silica films were used as controls. Cell metabolic activity was evaluated at days 1, 7, 14, and 21 using the alamar blue method (Invitrogen, USA),

following the parameters suggested by the manufacturer. ALP activity, an early indicator of bone cell differentiation, was measured in the medium at days 1, 7, 14 and 21 by mixing an aliquot of medium with p-nitrophenyl phosphate (Sigma, USA) at a 1:1 (v/v) ratio (Ali, Rowe *et al.,* 1996). This mixture was incubated for 15 minutes at room temperature and then the light absorbance was quantified at 405 nm to estimate the amount of dephosphorylated product relative to TCPS.

Changes in cell morphology were evaluated via fluorescence microscopy. For this, the cells were first fixed with 10% formalin for 30 minutes. F-actin filaments and cell nuclei were then stained with Alexa 488-phalloidin (Invitrogen) and PI-RNase (BD Biosciences), respectively. ImageJ was used to characterize a number of geometrical parameters (at t= 1 and 7 days), including nuclei alignment and aspect ratio.

### c. Statistical analysis

All the experiments were run in triplicate. A one-way ANOVA was used to detect significant differences between the groups. Error bars in the plots represent the standard error mean. All other numerical data are presented as box-whisker plots.

## 3. RESULTS AND DISCUSSION

The ability of the microimprinting method (Figure 45a) to transfer a specific texture from the stamp on to the silica film was first confirmed qualitatively using an interdigitated positive and negative line pattern with different dimensions. Figure 45b shows that the surface microfeatures were effectively copied by the silica. The post-sintering contraction of these micropatterns has been found to be ~11% (Pelaez-Vargas, Gallego-Perez *et al.,*

2010). No significant geometrical changes were seen, and the surface was free of defects.

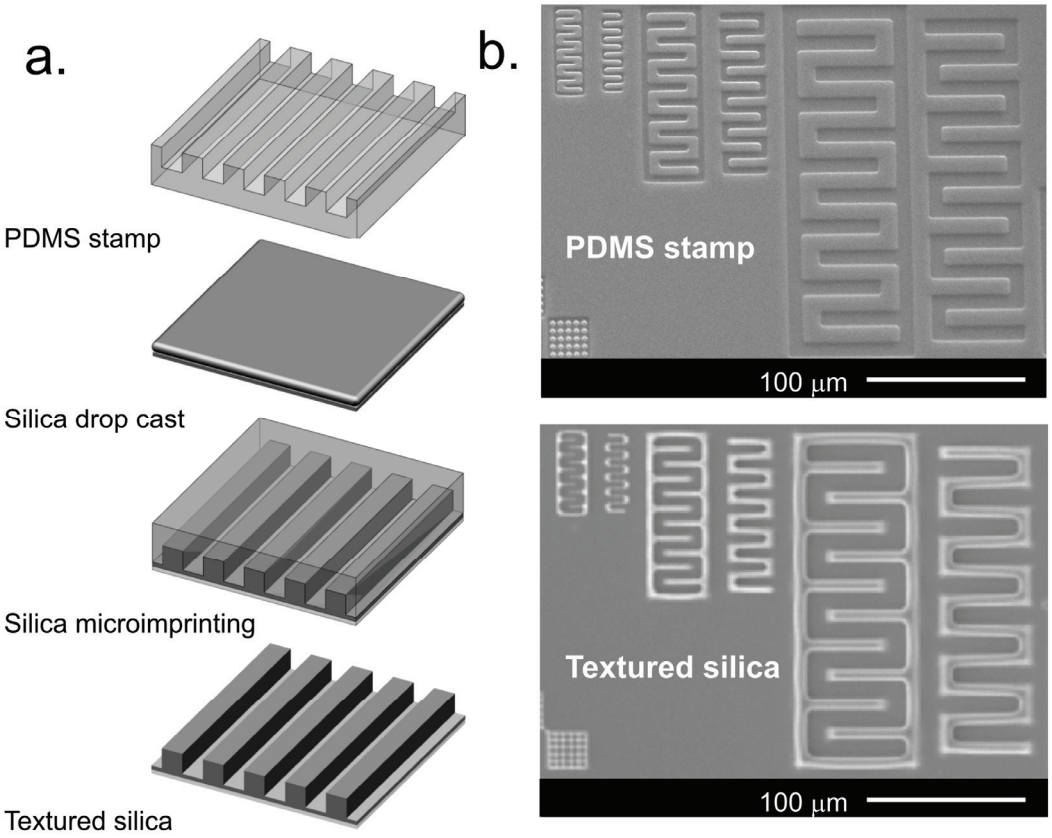



Figure 45. Soft lithography

(a) Depiction of the method used to micropattern thin silica films. Microimprinting was conducted at ~5.5 kPa for 2 hours. (b) PDMS stamp and (c) silica film with an interdigitated surface pattern.

Figure 46 shows SEM micrographs of the anisotropic silica patterns used in the cell culture experiments. Overall, the predesigned line width and interspacing were also successfully maintained between the PDMS stamp and their silica thin films.

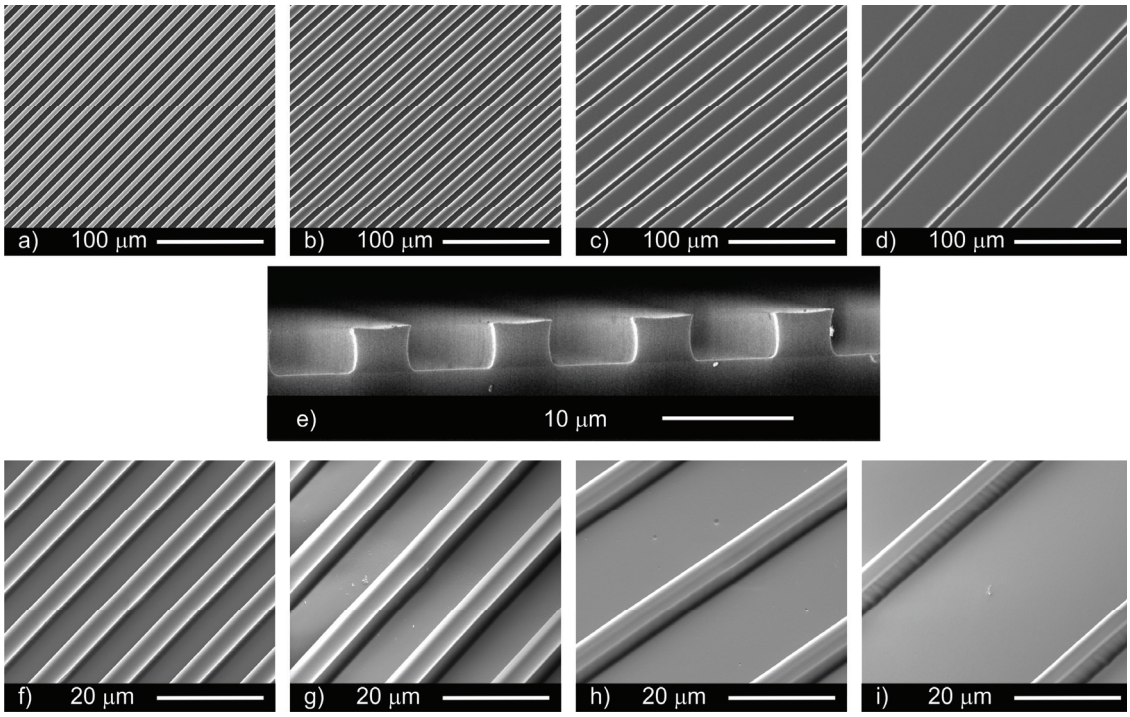



Figure 46. SEM Images of PDMS molds and micropatterned silica coating with different spacings.

PDMS stamps used to imprint the anisotropic silica patterns (a, d). Cross-section (e). (f-i) SEM images showing the microtextured silica surfaces in greater detail.

Human osteoblast-like cells were able to adhere, spread and propagate on both the patterned and control surfaces (Figure 47a). Cell growth was markedly guided by the surface microfeatures in terms of cell shape, alignment, elongation and propagation. Cell alignment on the microtextures was calculated based on the angle formed between the long axis of the nucleus and that of the lines on the silica patterns (Figure 47b). Most of the nuclei on the 5x5 and 5x10 μm patterns exhibited a high degree of alignment with the topography, with average angles below 7° after 1 and 7 days of culture. Although the F-actin cytoskeleton of cells cultured on 20 and 45 μm interspacing patterns appeared to be aligned with the topography (Figure 47b), the nuclei tended to present less acute angles. In addition, the nuclei of cells cultured on the microtextured silica had a tendency to be significantly more elongated compared to the control flat surfaces, as confirmed by the aspect ratio measurements (Figure 47b). Such orderly growth pattern could

potentially promote guided cell colonization of the implant surface *in vivo* (e.g., circumferentially ascending), which could be crucial for averting peri-implant tissue recessions that may lead to implant complications.

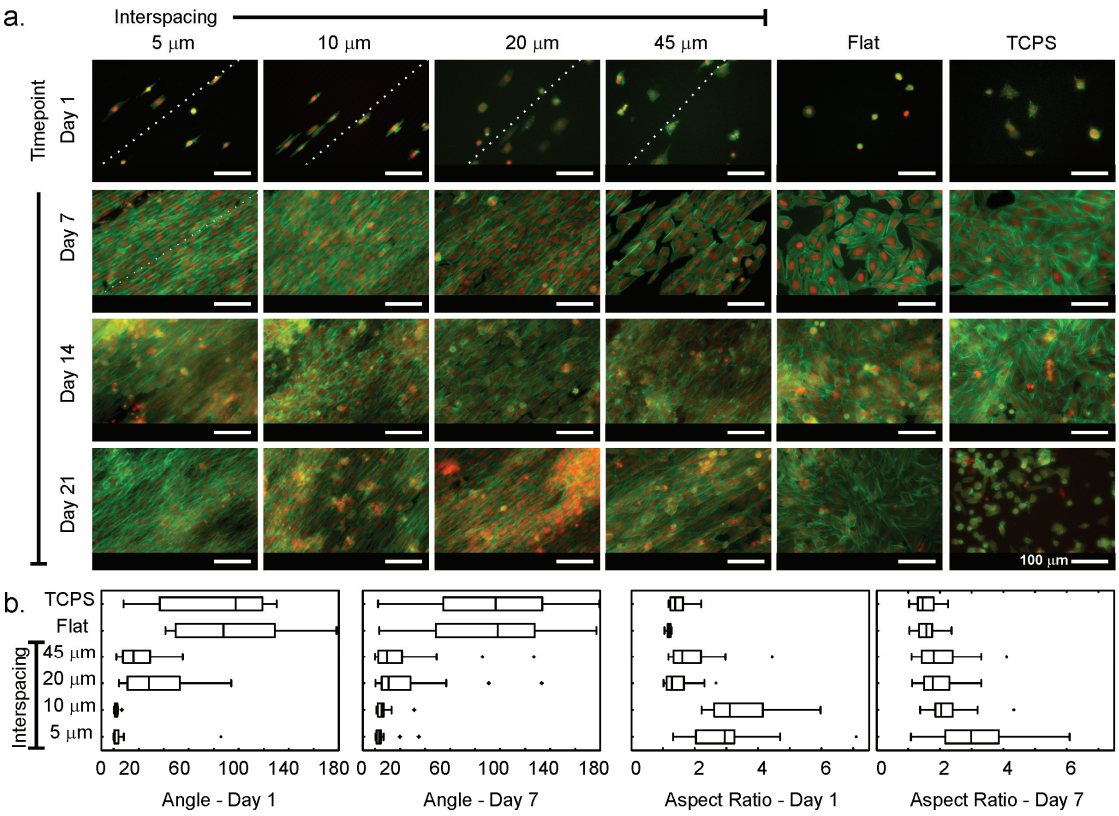



Figure 47. Fluorescence micrographs of human osteoblast-like cells.

a) Cells cultured on micropatterned silica coatings with 5 μm, 10 μm, 20 μm and 45 μm intespacing, flat silica, and TCPS for 1, 7, 14 and 21 days (F-actin stained green, and cell nuclei stained red). b) Box whisker plots of the (g, h) angle of nuclei alignment, and nuclei aspect ratio after 1 and 7 days of culture. Bar scale = 100 μm.

At early time points (i.e., 1 and 7 days of culture), the cell activity on the silica surfaces (patterned and flat) was comparable to TCPS controls, thus confirming the cytocompatibility of these surfaces (Figure 48a). After 14 and 21 days, the cells cultured on flat silica samples continued to exhibit activity levels similar to TCPS controls; however, cell cultures on anisotropic topographies were prone to present a significantly higher activity relative to flat silica surfaces, with denser (5 μm and 10 μm interspacing) textures having

a more pronounced effect (*p<0.01*) compared to less dense (20 μm and 45 μm interspacing) patterns (*0.05<p<0.1*). Changes in cell activity reflected combined variations in viability, proliferative and metabolic capacities.

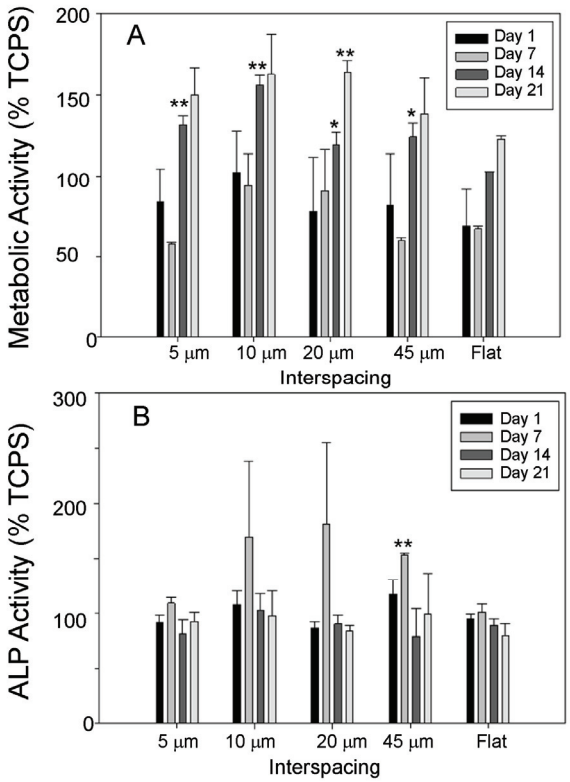



Figure 48. Results of Resazurin assay and ALP Activity

Plots of (a) cellular activity and (a) ALP activity in the medium. Asterisks indicate comparisons between the textured and flat surface at a given time point (* p<0.05** 0.05<p<0.1).

ALP activity has been widely accepted as a marker for bone cell functionality. Expression levels are typically higher during extracellular matrix deposition (Popat, Leoni *et al.*, 2007). Compared to TCPS controls, our results suggest that the ALP activity in the medium was not negatively impacted by the silica surfaces (Figure 48b). Nevertheless, the specific effect of the anisotropic microtextures on the expression/activity levels of this enzyme was not as marked, in comparison to the effect on the overall cellular activity of the culture (Figure 48a). Only less dense topographies (i.e., 5x45 μm) resulted in an early significant increase in ALP activity relative to their flat counterparts.

Different topography-mediated mechanisms have been proposed to influence bone cell responses, which ultimately lead to enhanced tissue-implant integration. The surface micro-nanotopography has been consistently shown to influence a host of important cellular processes (e.g. adhesion, propagation, proliferation, orientation, migration, differentiation, reactivity to hormones/growth factors) (Flemming, Murphy *et al.*, 1999; Martínez, Pradíes *et al.*, 2007). As of now, it is still debatable whether bone cells have direct interactions with the implant surface *in vivo* (Davies, 2003); however, there is a growing amount of evidence suggesting that surface micro-nanofeatures could potentially play a role in improving the clinical outcome of endosseous implants, presumably by inducing favorable interactions with bone cells and/or blood components (e.g. cells, proteins, minerals) (Brinker, Ashley *et al.*, 1996; Trisi, Lazzara *et al.*, 2003; Albrektsson and Wennerberg, 2004).

Our results indicated that cell morphology/spreading was greatly impacted by the surface microtexture. This could influence important factors such as cell alignment, migration, implant surface colonization, and function (Dike, Chen *et al.*, 1999; Flemming, Murphy *et al.*, 1999; Charest, Bryant *et al.*, 2004; Martínez, Pradíes *et al.*, 2007). Some studies have reported edge-localized formation of focal adhesions by rat osteoblasts on microgrooved surfaces (Matsuzaka, Walboomers *et al.*, 2003). Topographical modulation of the spatial distribution of focal adhesions is thought to lead to integrin-mediated intracellular signaling that may give rise to some of the observed cell behaviors on textured surfaces (Mendonça, Mendonça *et al.*, 2008). Integrin receptors have been postulated to transmit signals to the cells about the surface topography through cytoskeletal and adaptor proteins. Such signals could result in increased cell proliferation or osteogenic activity (Ogawa and Nishimura, 2003; Zinger, Zhao *et al.*, 2005; Wang, Zhao *et al.*, 2006).

Although in our case all the anisotropic surfaces consistently promoted increased cellular activity, with denser topographies resulting in more enhanced activity levels, the expression/activity of bone-related proteins was only augmented on the least dense topography. This differential effect could presumably be indirectly attributed to texture-mediated modulation of cell geometry/spreading (e.g., nuclei/cytoskeleton alignment and elongation) and spatiotemporal control of focal adhesion formation.

Some studies have also suggested that osseointegration is a function of the initial interactions that occur between the implant surface and different blood components. Platelet adhesion and activation has been found to increase on textured surfaces (Metroke, Parkhill *et al.*, 2001), which is expected to enhance endosseous integration due to the formation of an osteoconductive/osteoinductive blood thrombus (Body, 1996; Zhong and Greenspan, 2000). Other reports proposed that texture-mediated enhanced osseointegration may result from the stabilization of such clot via mechanical interlocking of fibrin fibers (Grew, Ricci *et al.*, 2008; Mendonça, Mendonça *et al.*, 2008). Finally, micro-nanostructured material surfaces have also been shown to provide a greater number of nucleation sites for the precipitation of minerals (e.g. Ca and P) from the blood plasma, which results in the formation of an amorphous apatite layer on the surface of the implant that could potentiate osseointegration (Kokubo, Kim *et al.*, 2004; Wang, Hsu *et al.*, 2008).

## 4. CONCLUSIONS

Sol-gel processing and soft lithography-based techniques were used synergistically to develop model ceramic glass textured surfaces that could be used as thin films on ceramic endosseous implants, to potentially improve the degree of tissue integration. Osteoblast-like cell responses were studied on

different types of anisotropic textures. The results indicated that the microtextures markedly modulated cell growth in terms of cell shape, orientation and propagation. In addition, the anisotropic surface microtextures also enhanced overall cell activity, and the expression/activity levels of bone-related proteins in some cases. Even though our main focus is on dental applications, the techniques herein described could be readily adapted to yield thin films with a wide range of textures on a myriad of materials of interest in implantology, thus providing improved implant surfaces for a much wider array of applications.

## 5. ACKNOWLEDGMENTS

This work was supported partially by the Portuguese Science and Technology Foundation (Scholarship FCT/SFRH/BD/36220/2007 and Grant No. FCT/PTDC/CTM/100120/2008 "Bonamidi") and NSF Nanoscale Science and Engineering Center fellowship (NSF, Grant No. EEC-0425626).

## REFERENCES

*To avoid duplicating information throughout the dissertation all references are presented in the general references section.*

# Manuscript 12. PORTLAND CEMENT POROUS SCAFFOLDS FOR LOAD-BEARING BONE TISSUE ENGINEERING APPLICATIONS

HIGUITA-CASTRO N, GALLEGO-PÉREZ D, **PELÁEZ-VARGAS A**, GARCÍA QUIROZ F, POSADA OM, LÓPEZ LE, SARASSA CA, AGUDELO-FLOREZ P, MONTEIRO FJ, HANSFORD DJ. Portland cement porous scaffolds for load-bearing bone tissue engineering application. ***J Biomed Mater Res. Part B - Appl Biomat.***

*Accepted August 2011.*

*Due that this is a secondary work made during the period as Visitang Scholar in the Dr. Hansford's Group at The Ohio State University. The information only is presented as abstract.*

***PORTLAND CEMENT POROUS SCAFFOLDS FOR LOAD-BEARING BONE TISSUE ENGINEERING APPLICATIONS***

HIGUITA-CASTRO N[1, 2], GALLEGO-PÉREZ D[1, 3], **PELÁEZ-VARGAS A**[4, 5], GARCÍA QUIROZ F [2], POSADA OM [2], LÓPEZ LE [2], SARASSA CA [6], AGUDELO-FLOREZ P [7], MONTEIRO FJ[4, 5], HANSFORD DJ*[1, 3].

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[5] Universidade do Porto. FEUP, DEMM, Porto, Portugal.
[6] Hospital Pablo Tobón Uribe, Medellín, Colombia.
[7] Instituto Colombiano de Medicina Tropical, Sabaneta, Colombia.

Modified Portland cement porous scaffolds with suitable characteristics for load-bearing bone tissue engineering applications were manufactured by combining the particulate leaching and foaming methods.

Polydimethylsiloxane was evaluated as a potential reinforcing material. The scaffolds presented average porosities between ~70-80% with mean pore sizes ranging from ~300 μm up to 5.0 mm. Non-reinforced scaffolds presented compressive strengths and elastic modulus values of ~2.6 and 245 MPa, respectively, whereas reinforced scaffolds exhibited an increase of ~60 and

80% on these parameters.

Portland cement scaffolds supported human osteoblast-like cell adhesion, spreading and propagation (t=1-28 days). Cell metabolism and ALP activity were found to be enhanced at longer culture intervals (t≥14 days) (Figure 49). These results suggest the possibility of obtaining strong and biocompatible scaffolds for bone repair applications from inexpensive yet technologically advanced materials such as Portland cement.

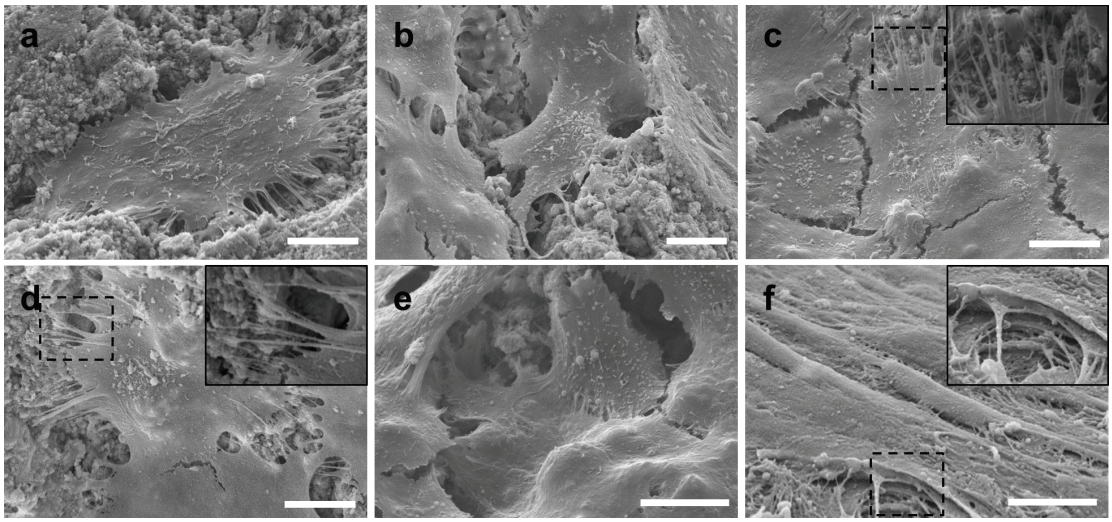



Figure 49. SEM micrographs of SaOs-2 cells cultured on a) Porus sample 2% $H_2O_2$ treated and b) porous samples reinforced treated with 2% $H_2O_2$ and 7% PDMS.

(a) PS-2 (Porus sample 2% $H_2O_2$) for 7 days, (b) PSR-2.7 for 14 days, (c) PS-2 and (d) PSR-2.7 for 21 days, and (e) PS-2 and (f) PSR-2.7 for 28 days. Scale bar = 10 μm. Insets in (c) and (d) show higher magnification images of the filopodia. Inset in (f) shows a multilayered lamellar-like structure, typically seen in longer term cultures.

## Abstract 15. HGF ADHESION ON MODIFIED SURFACES OF ZIRCONIA BASED FRAMEWORK CORES

**PELÁEZ-VARGAS A**, RESTREPO LF, FERNANDES MH, FERRAZ MP, HANSFORD DJ, MONTEIRO FJ. HGF Adhesion on Modified Surfaces of Zirconia Based Framework Cores. Proceedings MRS FALL Meeting SS5.29. Boston (MA). 2009.

***HGF ADHESION ON MODIFIED SURFACES OF ZIRCONIA BASED FRAMEWORK CORES***

**PELÁEZ-VARGAS A**[1,2], RESTREPO LF[3], FERNANDES MH[4], HANSFORD D [2], MONTEIRO FJ[1]

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Introduction: Zirconia ceramics, in combination with CAD/CAM techniques, present an appealing choice for partially fixed restorations involving molar, due to their high mechanical performance and aesthetic appearance. However, restorations in anterior areas are a challenge because the pigments, additives and porcelains used may modify any roughness features and the biological response of the restorations. With this in mind, our objective is to compare the adhesion, morphology and proliferation of Human Gingival Fibroblast (HGF) cells cultured on surface modified zirconia based framework cores (ZBFC).

Methods: Thirty-six ZBFC were produced using CAD/CAM technology (Procera) (Figure 50) and divided into three groups (n=12): (AZBFC) Aluminum Oxide Veenering Porcelain (NobelRondo) on ZBFC; (PZBFC) Pigment Ceramic Layer (NobelRondo) on ZBFC; and ZBFC without modification as a control group.
AZBFC samples were prepared by a multilayer technique including baseline, dentin porcelain, enamel porcelain, and glass layers application. In the PZBFC group, a single body stain Zirconia layer was applied on ZBFC. All thermal cycles followed the manufacturer instructions.

SEM/EDS and AFM were used for chemical and surface characterizations. In vitro biological evaluation used HGF cells, cultured according to a standardized protocol for four days. Morphology and proliferation were evaluated by optical microscopy.

Results: Elemental analysis by EDS showed differences among the surfaces of the three groups evaluated in agreement with different applied layers. AFM analysis showed the following order of roughness values: ZBFC>PZBFC>AZBFC.

HGF presented a normal morphology on all surfaces, at 4h, 24h and 4 days. HGF proliferation using MTT assay did not reveal any statistically significant differences at intergroup analysis and showed statistical significant differences for 24h and 4 days at intragroup analysis.

Conclusions: These preliminary studies revealed that ZBFC modified with body stain or alumina veneering porcelain did not induce morphological or proliferative changes on HGF cultures.

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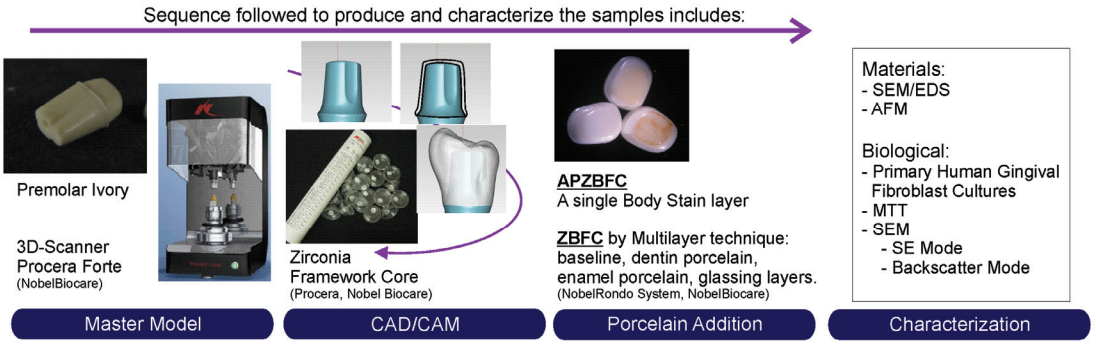



Figure 50. Flow diagram from the ivorine master model, trought by CAD/CAM phase, porcelain adition and matrials characterization

## Abstract 16. MICRO-STRUCTURED SILICA COATINGS WITH HA PARTICLES USING A PDMS STAMP

**PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, FERNANDES MH, HANSFORD DJ, MONTEIRO FJ. Micro-Structured Silica Coatings with HA Particles Using a PDMS Stamp. VV8.9. Proceedings MRS FALL Meeting SS5.29. Boston (MA). 2009.

***MICRO-STRUCTURED SILICA COATINGS WITH HA PARTICLES USING A PDMS STAMP***

**PELÁEZ-VARGAS A** [1], GALLEGO-PÉREZ D [2], FERNANDES MH [3], HANSFORD DJ [2], MONTEIRO FJ [1].

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It is well known that titanium is the gold standard for oral implantology. However, in many clinical cases its appearance is not ideal for patients with thin gingival tissues. Yttrium stabilized zirconia (Y-TZP) and zirconia toughened alumina (ZTA) are alternative materials that have excellent aesthetic performance but slow osseointegration. The implementation of bioactive coatings could be used as a strategy to address this problem. In this work, a technique that includes silica sol-gel chemistry and soft lithography technology was developed to produce micropatterned silica coatings with hydroxyapatite nanoparticles (nanoHA).

NanoHA was prepared following a standardized methodology in our group. Silica sol was obtained using alkoxides (TEOS and MTES). A meta-stable solution of silica with nanoHA particles was prepared. A microtexturing technique using previously patterned PDMS stamps was optimized using a multilevel resolution mask with feature sizes ranging from 500nm to 20 μm.

Morphological characterization before and after sintering was conducted via SEM. The coatings were then sterilized and cultured with MG63 human osteoblast-like cells using a standardized protocol at two time points (4h and 24h). Adhesion and cell morphology were evaluated using SEM and LSCM (Figure 51).

Crack-free micropatterned silica coatings with nanoHA particles were successfully obtained. SEM and LSCM images showed cell alignment in the direction of the micropatterns. These preliminary results showed that micropatterned bioactive silica coatings on dental ceramics could find applications in the fabrications of aesthetic oral implants.

The PhD grant FCT/SFRH/BD/36220/2007 is acknowledged.

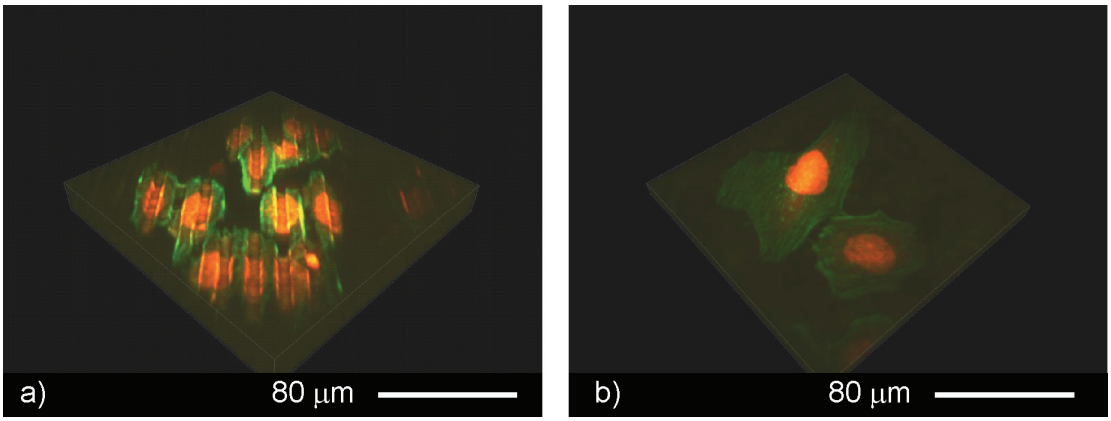



Figure 51. CLSM images of SaOs-2 cells on a) micropatterned (Lines 5x5 μm) and b) flat silica thin films

## Abstract 17. SILICA COATINGS WITH PORTLAND CEMENT PARTICLES FOR DENTAL IMPLANT SURFACES BASED ON SOL-GEL MICROMOLDING

**PELÁEZ-VARGAS A**, GALLEGO-PÉREZ D, HIGUITA-CASTRO N, FERRELL N, HANSFORD D, MONTEIRO FJ. Silica coatings with Portland cement particles for dental implant surfaces based on sol-gel micromolding. Proceeding of Biomedical Engineering Society (CD PS 10A-23). 2009.

***SILICA COATINGS WITH PORTLAND CEMENT PARTICLES FOR DENTAL IMPLANT SURFACES BASED ON SOL-GEL MICROMOLDING***

**PELÁEZ-VARGAS A** [1], GALLEGO-PÉREZ D [2], HIGUITA-CASTRO N [2], FERRELL N [2], HANSFORD D [2], MONTEIRO FJ [1].

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Particles have been used to bioactivate dental devices using several chemistries, deposition processes and sizes. Apatites are the most studied group of coating materials to improve the biological response during osseointegration. However, longitudinal clinical studies show contradictory success rate between coated and uncoated implants.
The purpose of this work was to develop a dual molding technique based in soft lithography to produce micropatterned silica coatings with carbonated Portland cement particles (CPCP).

CPCP were prepared by a standard process. Soft lithography was used to create particle containing micropatterned silica thin films on a model glass surface. Two different methods were tested (single and dual molding). The samples were sintered at 500$^{o}$C for 60min and examined by SEM.

CPCP particles showed a bimodal-like size distribution (D50= 5.9 μm). CPCP revealed a bioactive behavior in an SBF test. A metastable solution was obtained using CPCP and silica sol as solid and liquid phases, respectively. Deposited films had thicknesses below 4 μm. Each component (particles and silica matrix) independently showed cytocompatibility using Human Osteosarcoma cell cultures. Coatings with ~5 μm microfeatures were

successfully reproduced over large areas. The dual molding technique provided better results (Figure 52).

Future work includes particle-loaded and micropatterned coatings on dental materials and extensive biological evaluation.

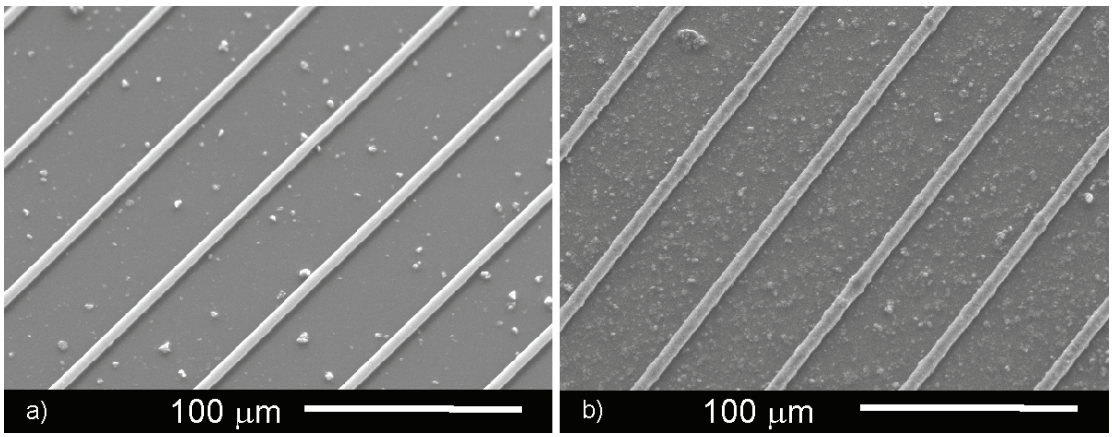



Figure 52. SEM Images of Anisotropic silica thin films (Interspacing = 45 μm) produced by two diferent stamping methods. a) Dual molding and b) single molding.

## Abstract 18. MICROASSEMBLY OF MULTIFUNCTIONAL BONE TISSUE ENGINEERING SCAFFOLDS

GALLEGO-PÉREZ D, MICHALEC D, **PELÁEZ-VARGAS A**, HIGUITA-CASTRO N, MONTEIRO FJ, MILLER MJ, HANSFORD DJ. Layer-by-Layer Microassembly of Multifunctional Bone Tissue Engineering Scaffolds. Proceeding Biomedical Engineering Society (CD - OP 9-3-12C). 2009.

*It is a secondary work made during the period as Visitang Scholar in the Dr. Hansford's Group at The Ohio State University.*

***LAYER-BY-LAYER MICROASSEMBLY OF MULTIFUNCTIONAL BONE TISSUE ENGINEERING SCAFFOLDS***

GALLEGO-PÉREZ D, MICHALEC D, **PELÁEZ-VARGAS A**, HIGUITA-CASTRO N, MONTEIRO FJ, MILLER MJ, and HANSFORD DJ.

Traditional scaffolds are made out of single materials and exhibit randomly distributed porosity. However, uncontrolled architecture could lead to necrotic cores due to limited waste/nutrient exchange. We present a soft lithography approach to fabricate scaffolds with controlled microstructure and chemistry in layers that are then assembled into a collective scaffold structure. Individual layers were manufactured from various biomedical polymers (PLGA, PCL, PCL/nanohydroxyapatite, PMMA, and PVDF). Heating and stamping parameters were developed to combine/assemble layers into scaffolds with robust attachments. The scaffolds presented a fully interconnected structure with 100 μm pores, in which layer geometry was based on an SU8 master. Testing in physiologic solutions showed that the structure was maintained for at least 4 weeks.

Preliminary experiments (Figure 53) with human osteosarcoma cells revealed that individual layer properties could modulate cellular responses (adhesion, organization and alignment). In addition, gold-coated PCL and piezoelectric PVDF layers were successfully combined to create electromechanically active scaffolds capable of delivering electrical and/or mechanical stimuli to the forming tissue. Initial testing on PVDF microstructures showed strains in the bone's physiological range at low voltages. Our fabrication method also allows for the production of scaffolds with unique layer permutations for different tissue engineering applications.

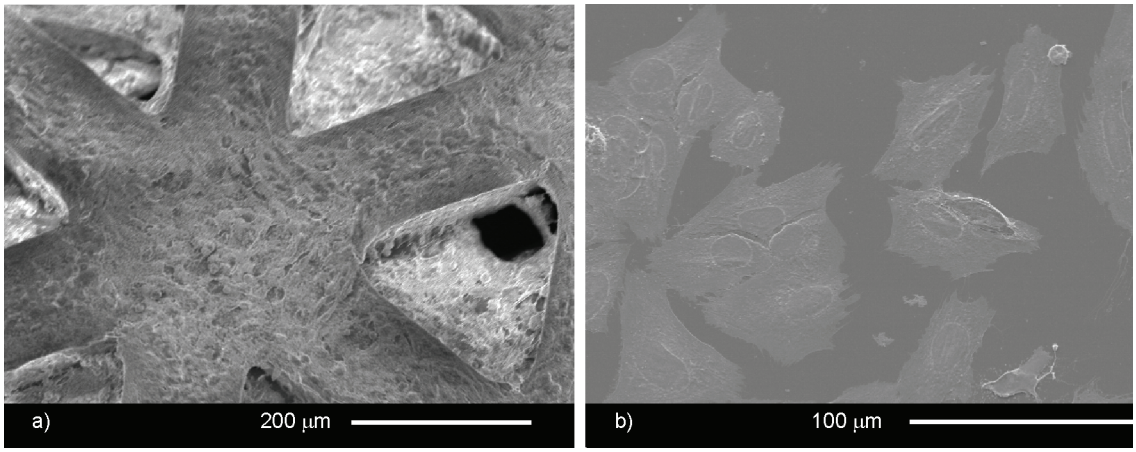



Figure 53. SEM images of multilayer scaffolds.

a) Scaffolds made out of single materials (PCL, PCL-PC, or PCL-HA) or multiple materials (e.g. PCL-PC or PCL-HA sandwiched between PCL layers, or PCL-PCLHA-PCLPC) were cultured with human osteoblast-like cells (SaOs-2) for 4 days. b) TCPS control surface.

# Abstract 19. MICRO-PATTERNING COMBINED WITH LIVE-CELL IMAGING: A TOOL FOR IN VITRO STUDIES OF DENTAL TISSUE GUIDED REGENERATION

PELÁEZ-VARGAS A[1,2], SAMPAIO P[3], GALLEGO-PÉREZ D[4], FERNANDES MH[5], HANSFORD D[4] AND MONTEIRO FJ[1,2] · *Micro-patterning combined with live-cell imaging: a tool for in vitro studies of dental tissue guided regeneration. Memories of I3S retreat. Retreat. Póvoa de Varzim. Portugal. 2011.*

***MICRO-PATTERNING COMBINED WITH LIVE-CELL IMAGING: a tool for in vitro studies of dental tissue guided regeneration***

**PELÁEZ-VARGAS A** [1, 2], SAMPAIO P [3], GALLEGO-PÉREZ D [4], FERNANDES MH [5], HANSFORD D [4] and MONTEIRO FJ [1, 2]

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Introduction: Cells derived from Human Oral Soft Tissues (i.e. gingiva, pulp, bone and periodontal ligament) are poorly characterized in terms of dynamic behavior during adhesion, spreading and proliferation stages on dental materials.

Classical approaches include in vitro characterization using video-register techniques on TCPS surfaces or other polymers that are not comparable with clinical surfaces. These limitations may be solved under an interdisciplinary approach based on live-cell imaging (ALM unit from IBMC) and microfabrication technologies (Sol-gel from Biocomposites group/INEB and Soft lithography from The Ohio State University).

Objective: To study the in vitro dynamic behavior of cells derived from human oral tissues (Human Pulp-derived MSCs) on micropatterned hard materials using time-lapse microscopy and digital image analysis.

Results: Preliminary results showed that Human Pulp-derived MSCs exhibit adhesion times modulated by interspacing of the silica micropatterned thin

films. All patterned surfaces (line-shape with 5, 10, 20, 45 µm interspacing) induced cellular alignment and directional movement, a behavior not observed on flat surfaces (Figure 54).

Conclusion: Time-lapse imaging and micropatterned hard surfaces produced by sol-gel/soft lithography technology are helpful in studying the dynamic behavior of Human Pulp-derived MSCs for guide tissue regeneration in dental implantology.

Relevance: The synergy between production of micropatterned hard surfaces and time-lapse imaging analysis may help in studying guided tissue regeneration of several tissues important in dentistry.

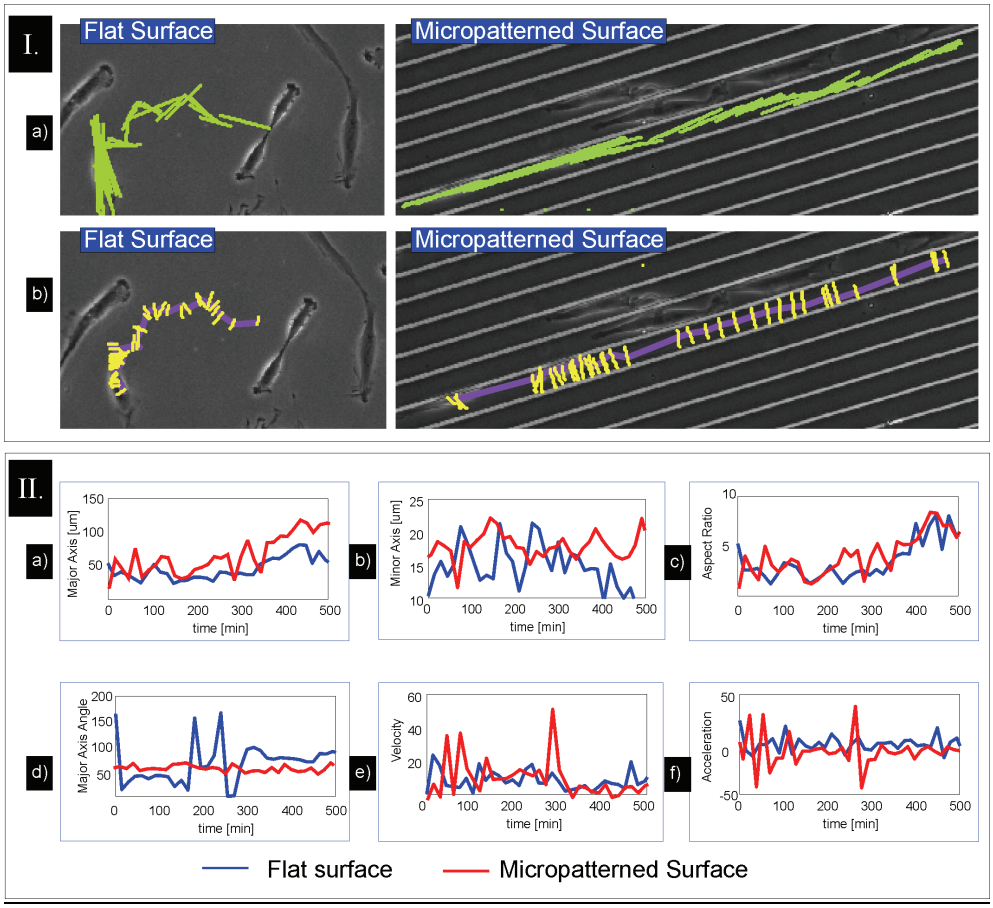



Figure 54. Motility behavior of individual Human Pulp-derived MSCs on silica flat and micropatterned surfaces.

Images from dark field microscopy (I) showed the accumulated behavior of the (a) major axis (green) and b) minor axis (yellow) and centroid (purpure) for each photogram (green). After a manual segmentation, the morphological information (II) was generated using Matlab in terms of size of axis (a-b), aspect ratio (c), major axis angle (d), velocity (e) and acceleration (f). Preliminary results shown a three times higher motility and directionality on micropatterned surfaces.

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